# MICROBIOLOGICAL STUDIES OF *STREPTOCOCCUS* SP. BIOVAR 1 (SYNONYM OF *ENTEROCOCCUS SERIOLICIDA* AND *LACTOCOCCUS GARVIEAE*), PATHOGENIC FOR RAINBOW TROUT (*ONCORHYNCHUS MYKISS*), AND RELATED SPECIES OF BACTERIA


**BY**

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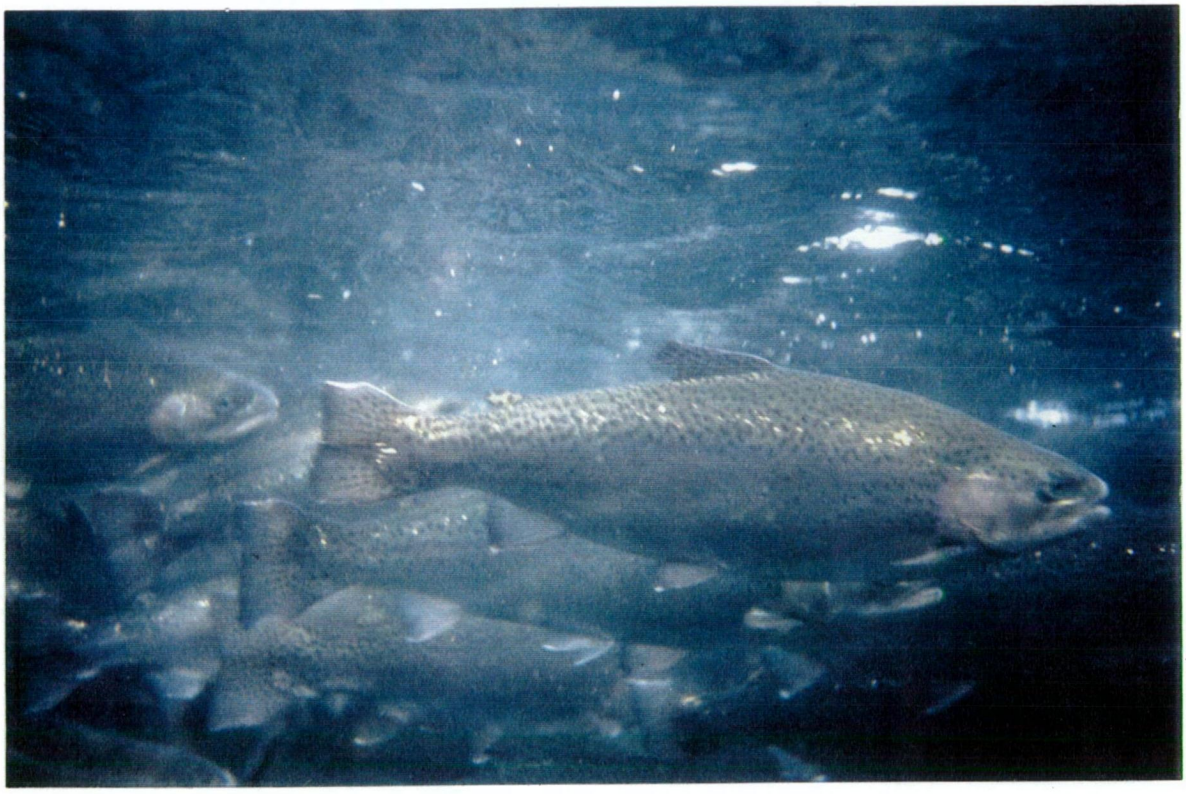

**Submitted in fulfilment of the requirements of the degree of**

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## STATEMENT

I, Tiina Hawkesford, declare that this thesis contains no material that has been accepted for the award of any other degree in any University or College, and to the best of my knowledge and belief, the thesis contains no material previously published or written by another person except when due reference is made in the text of the thesis.

Signed,

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## ABSTRACT

A specific Gram-positive coccus, which was given the epithet *Streptococcus* sp. biovar 1, was first isolated from diseased farmed rainbow trout (*Oncorhynchus mykiss*) in Tasmania in 1988. The preliminary identification was based on the morphological, physiological and biochemical characteristics of the Tasmanian isolates. However, subsequent studies in Japan and Tasmania indicated that these organisms were more closely related to the genus *Enterococcus*, especially the fish-pathogenic bacterium known as *Enterococcus seriolicida*, than to the genus *Streptococcus*. Further investigation has indicated that these fish pathogenic isolates are identical to the species *Lactococcus garvieae* isolated from cattle.

In December 1993, when this study commenced, the source of *Streptococcus* sp. biovar 1 (as it was then known) in Tasmania was unknown, so an environmental survey of potential sources was undertaken with the view to determining the initial source of the organism and providing a basis for future control of the disease. The 61 organisms isolated during this survey, as well as ten Australian isolates of *Streptococcus* sp. biovar 1, two strains of *Enterococcus seriolicida* and two strains of *Lactococcus garvieae*, were characterised phenotypically using morphological, physiological, biochemical and immunological techniques. Random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) studies were then undertaken on the ten Australian isolates of *Streptococcus* sp. biovar 1, two strains of *Enterococcus seriolicida*, one strain of *Lactococcus garvieae* and 18 of the environmental isolates to investigate the possibility of any genetic similarity.

In vivo pathogenicity to rainbow trout (*Oncorhynchus mykiss*) was also studied to compare the virulence of the Australian isolates of *Streptococcus* sp. biovar 1 with that of *Lactococcus garvieae*. This was performed as two separate experiments. The first experiment was to establish the $LD_{50}$ of one of the Tasmanian isolates, including examination of histopathology to evaluate the tissue tropism of the organism. The second experiment was to passage *Lactococcus garvieae* isolated from a case of bovine mastitis through rainbow trout. Two techniques, direct kidney tissue transfer and culture and re-isolation, were used to see if the organism could produce disease.

The results of all these studies showed that the Australian isolates (*Streptococcus* sp. biovar 1) were closely related to, if not identical with, both *Lactococcus garvieae* and organisms previously named *Enterococcus seriolicida.* The results also showed that none of the isolates from the environment were phenotypically or genotypically the same as the Australian isolates or *Lactococcus garvieae.*

Therefore, *Streptococcus* sp. biovar 1 should probably be reclassified as *Lactococcus garvieae,* and, because it is known to be the cause of streptococcosis in many countries around the world, it must now be considered a major fish pathogen worldwide.

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## LIST OF ABBREVIATIONS

| Reaction | Type | Code |
|---|---|---|
| Haemolysis | alpha | α |
| | beta | β |
| | gamma | γ |
| Fermentation | acid | a |
| | different | d |
| | weak | w |
| | negative | - |
| Tests | positive | + |
| | negative | - |
| | variable | V |
| Lancefield antigen | group D | D |
| | group N | N |
| Agglutination | positive | pos |
| | negative | neg |
| | weak | w |
| Other | not documented | ND |
| Sensitivities | sensitive | S |
| | resistant | R |

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## *CHAPTER ONE*: LITERATURE REVIEW

### 1.1 Overview of lactic acid-producing bacteria

The lactic acid-producing bacteria now consist of the genera *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Tetragenococcus* and *Vagococcus* (Axelsson, 1993). These bacteria grow anaerobically and most are not sensitive to oxygen, but can grow in its presence as well as its absence and are, therefore, termed aerotolerant anaerobes (Madigan *et al.* 1997). Most of the lactic acid group of organisms display homofermentative fermentation, which means that the fermentation of glucose or other sugar results in the production of a single product, lactic acid (Madigan *et al.* 1997). A great deal of taxonomic research has been performed on these bacteria over the last hundred years by numerous scientists, including those working in the medical, veterinary, dairy and microbiology fields (Logan, 1994). The fact that the fields of research were quite diverse is probably responsible for the fact that, until recently, there has been little cohesion in the taxonomy of this group of organisms. In order to try and simplify the development of the taxonomy of this complex group of organisms, the taxa will be mainly discussed in chronological order. Some differential tests are summarised in Table 1.1.

**Table 1.1 Differential tests for some genera of the Streptococcaceae (Carson *et al.* 1993)**

| Test | *Streptococcus* | *Enterococcus* | *Lactococcus* |
|---|---|---|---|
| Growth at 10 °C | - | + | + |
| Growth at 45 °C | V | +[2] | - |
| Growth in 6.5% NaCl | - | +[3] | V |
| PYR[1] hydrolysis | -[4] | + | -[4] |
| Bile-esculin reaction | -[5] | + | V |
| Group D antigen | - | V | - |

[1] L-pyrrolidonyl-β-naphthylamide

[2] *E. malodoratus* is negative

[3] *E. pseudoavium* is negative

[4] *S pyogenes* and *L. garvieae*

[5] Some exceptions

### 1.1.1 Overview of the genus *Streptococcus*

In 1884, Rosenbach proposed the species *Streptococcus pyogenes* (Skinner & Quesnel, 1978; Schleifer & Kilpper-Bälz, 1987; Logan, 1994) and *Staphylococcus aureus* as the type species of their respective genera, but while the staphylococci remained taxonomically static for many years, the great diversity of the streptococci was recognised early and attempts to satisfactorily classify these organisms were soon made (Logan, 1994). According to Rosenbach in 1884, the genus *Streptococcus* consisted of catalase-negative, Gram-positive cocci which were arranged in pairs or chains and were usually described as facultatively anaerobic (Schleifer & Kilpper-Bälz, 1987). The term "facultatively anaerobic" to describe all species of *Streptococcus* is not strictly correct as most of the species are now considered to be aerotolerant anaerobes (Madigan *et al.* 1997).

In 1906, Andrewes & Horder, using morphological observations and biochemical tests, recognised eight groups of streptococci from humans, milk and air, and then in 1919 Orla-Jensen, using a wider range of tests, recognised ten groups, the pathogenic strains being grouped as *S. pyogenes* for want of more information (Skinner & Quesnel, 1978; Schleifer & Kilpper-Bälz, 1987; Logan, 1994). In 1933 a serological test was proposed to differentiate streptococci into several groups by a precipitin test (Lancefield, 1933). Initially, the most important or pathogenic groups in this series were groups A to E and later N. The results of these findings are summarised in Table 1.2. Following the detection of the Lancefield group-specific antigens, the classification and identification of streptococci has relied heavily on this serological grouping technique (Schleifer & Kilpper-Bälz, 1987).

**Table 1.2 Summary of the differentiation of human and other groups of haemolytic streptococci (Lancefield, 1933)**

| Antigenic group | Main sources or diseases |
|---|---|
| A | Scarlet fever & throat infections in man |
| B | Bovine mastitis |
| C | Guinea pig lymphadenitis & bovine mastitis |
| D | Cheese |
| E | Cow (milk) |

In 1937 Sherman suggested that the initial division of strains into groups, according to growth temperatures, reducing ability, and resistance to heat, alkalinity and methylene blue, would make interpretation of other tests easier. He thus arrived at four groups comprising pyogenic, viridans and lactic streptococci and enterococcus. His findings correlated quite well with the Lancefield grouping system, as can be seen in Table 1.3.

**Table 1.3 Divisions of the streptococci (Sherman, 1937)**

| Pyogenic | Grp | Viridans | Grp | Lactic | Grp | Enterococcus | Grp |
|---|---|---|---|---|---|---|---|
| *S. pyogenes* | A | *S. salivarius* | (K) | *S. lactis* | N | *S. faecalis* | D |
| *S. mastiditis* | B | *S. equinus* | D | *S. cremoris* | N | *S. durans* | D |
| *S. equi* | C | *S. bovis* | D | | | *S. liquefaciens* | D |
| | | | | | | *S. zymogenes* | D |

Garvie *et al.* (1981) studied the properties of some isolates of streptococci that grew at 10°C, but not at 45°C, including *Streptococcus lactis, Streptococcus cremoris* and *Streptococcus raffinolactis,* and concluded that some of the isolates, including one that was isolated from a case of bovine mastitis, were genetically different from the known strains. The bovine mastitis isolate has since been named by Collins *et al.* (1983) as *Streptococcus garvieae.*

### 1.1.2 Overview of the genus *Enterococcus*

Thiercelin and Jouhaud, in 1903, first named the genus *Enterococcus.* The genus has only recently been properly revived in 1984 by Schleifer and Kilpper-Bälz, for bacteria previously named *Streptococcus faecalis* and *Streptococcus faecium* (Schleifer & Kilpper-Bälz, 1984; Devriese *et al.* 1993). Sherman (1937) classified *Streptococcus faecalis* and *Streptococcus durans* in his "enterococcus" division of the streptococci, but *Streptococcus faecium* was not designated a separate species, because he considered it to be identical to *Streptococcus faecalis.*

Sherman (1937) excluded *Streptococcus bovis* and *Streptococcus equinus* from his enterococcus classification even though he demonstrated the group D antigen in them. Although the group D antigen test is an important diagnostic tool for the *Enterococcus* species, not all species of *Enterococcus* possess this antigen. Those species which do not possess the group D antigen now include *E. avium, E. pseudoavium, E. cecorum, E. dispar, E. saccharolyticus* and *E. seriolicida* (Kusuda

*et al.* 1991, Holt *et al.* 1994). However, as will be discussed later, *Enterococcus seriolicida* should probably no longer be regarded as a member of the genus *Enterococcus*. Also both the group D and N antigens (the latter associated with the "lactic' species) are, in fact, glycerol techoic acids located between the cell wall and membrane, whereas other Lancefield antigens are rhamnose-containing polysaccharides of the cell wall (Logan, 1994).

More recently, in 1982, Bridge & Sneath recognised 21 species from the enterococcal, viridans, lactic, thermophilic pyogenic and pneumococcal groups. They also found that *Aerococcus, Gemella, Leuconostoc* and *Pediococcus* were close relatives of the streptococci (Bridge & Sneath, 1983). Since the revival of the genus

**Table 1.4 List of *Enterococcus* species described up to 1993 (Devriese *et al.* 1993)**

| Present name | Author | Previous genus |
|---|---|---|
| *Enterococcus avium* | Collins *et al.* 1984 | *Streptococcus* |
| *Enterococcus casseliflavus* | Collins *et al.* 1984 | *Streptococcus* |
| *Enterococcus cecorum* | Williams *et al.* 1989 | *Streptococcus* |
| *Enterococcus columbae* | Devriese *et al.* 1990 | new |
| *Enterococcus dispar* | Collins *et al* 1991 | new |
| *Enterococcus durans* | Collins *et al.* 1991 | *Streptococcus* |
| *Enterococcus faecalis* | Schleifer & Kilpper-Bälz, 1984 | *Streptococcus* |
| *Enterococcus faecium* | Schleifer & Kilpper-Bälz, 1984 | *Streptococcus* |
| *Enterococcus flavescens* | Pompei *et al.* 1992 | new |
| *Enterococcus gallinarum* | Collins *et al.* 1984 | *Streptococcus* |
| *Enterococcus hirae* | Farrow & Collins, 1985 | new |
| *Enterococcus malodoratus* | Collins *et al.* 1984 | *Streptococcus* |
| *Enterococcus mundtii* | Collins *et al.* 1986 | new |
| *Enterococcus pseudoavium* | Collins *et al.* 1989 | new |
| *Enterococcus raffinosus* | Collins *et al.* 1989 | new |
| *Enterococcus saccharolyticus* | Rodrigues & Collins, 1990 | *Streptococcus* |
| *Enterococcus seriolicida* | Kusuda *et al.* 1991 | new |
| *Enterococcus solitarius* | Collins *et al.* 1989 | new |
| *Enterococcus sulfureus* | Martinez-Murcia & Collins, 1991 | new |

*Enterococcus* by Schleifer and Kilpper-Bälz in 1984, chemotaxonomic and phylogenetic studies have resulted in the addition of 17 other species to this genus.

Several of these have been transferred from the genus *Streptococcus*, and others have been newly described (Table 1.4) (Devriese *et al.* 1993).

### 1.1.3 Overview of the genus *Lactococcus*

One of the several changes in the taxonomy of the genus *Streptococcus*, that has occurred in the past few years, is the establishment of the genus *Lactococcus* (Schleifer *et al.* 1985). This includes bacteria formerly known as the lactic group of streptococci, consisting of *Streptococcus lactis, Streptococcus cremoris* and, in some texts, *Streptococcus diacetylactis*. When the genus *Lactococcus* was established, some species originally classified under the genus *Streptococcus* were transferred to the new genus and renamed *Lactococcus garvieae, Lactococcus plantarum* and *Lactococcus raffinolactis* (Schleifer *et al.* 1985). Some of the original species were made subspecies of *Lactococcus lactis*, including *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* (Schleifer *et al.* 1985).

The difficulty of phenotypic identification by conventional means occurs because of the scarcity of tests for distinguishing between lactococci and enterococci. Some strains of both genera show growth in broth containing 6.5% NaCl, a positive PYR (L-pyrrolidonyl-β-naphthylamide) test and hydrolyse bile esculin. Some studies indicate that lactococci can be differentiated from enterococci by their growth at 10°C, but not at 45°C (enterococci grow at both temperatures) and failure to have physiological or serological characteristics identical to *Enterococcus* spp. (Elliott *et al.* 1991; Schleifer & Kilpper-Bälz, 1987).

### 1.1.4 Techniques for phylogenetic studies of "lactic acid" bacteria

The most commonly used technique for differentiating streptococci, enterococci and lactococci is the serological Lancefield grouping (Lancefield, 1933), but as there are many catalase negative cocci that do not possess a group-specific antigen, it cannot be relied on as the sole method of differentiation (Schleifer & Kilpper-Bälz, 1987; Logan, 1994). Therefore, methods such as nucleic acid hybridisation, 16S rRNA oligonucleotide cataloguing and chemotaxonomic analyses have more recently led to significant changes in the classification of Gram-positive, catalase-negative,

facultatively anaerobic (or aerotolerant anaerobic) cocci previously included in the genus *Streptococcus* (Schleifer & Kilpper-Bälz, 1987).

Because the taxonomy of *Enterococcus* sp. had not been completely defined, DNA-DNA hybridisation and contour clamped homogenous electric field (CHEF) electrophoresis have been used to identify some enterococci to species level. However, these techniques may also be of some use for species differentiation amongst the enterococci (Donabedian *et al.* 1995). The "lactic", or Lancefield group N, streptococci have recently been extensively reviewed by nucleic acid hybridisation and rRNA sequencing (Schleifer *et al.* 1985). The results demonstrate that the "lactic" streptococci form a phylogenetically distinct group, for which the genus *Lactococcus* has been described (Schleifer *et al.* 1985; Williams & Collins, 1992; Austin & Austin, 1993). Nucleic acid hybridisation studies and immunological relationships of superoxide dismutase demonstrated that *Streptococcus lactis* (and its subspecies), *Lactobacillus xylosus, Lactobacillus hordiniae, Streptococcus garvieae, Streptococcus plantarum* and *Streptococcus raffinolactis* are closely related to each other, but not to other streptococci. Therefore, it was proposed that these taxa be transferred to a new genus *Lactococcus* gen. nov. as *Lactococcus lactis* subsp. *lactis* (including former *S. lactis* subs. *diacetilactis* and *Lactobacillus xylosus*), *L. lactis* subsp. *cremoris*, *L. lactis* subsp. *hordiniae*, *L. garvieae*, *L. plantarum* and *L. raffinolactis* (Schleifer *et al.* 1985; Schleifer & Kilpper-Bälz, 1987). Colony hybridisation with 23S rRNA-targeted oligonucleotide probes has also reliably distinguished species of *Lactococcus* and *Enterococcus* species within mixed cultures (Betzl *et al.* 1990).

### 1.2 Diseases of fish caused by Gram-positive cocci

#### 1.2.1 "Lactic acid" bacteria associated with fish disease

The lactic acid-producing bacteria, including species of the genera of *Streptococcus, Enterococcus , Lactococcus, Carnobacterium,* and *Lactobacillus* (Austin & Austin, 1993) and *Vagococcus*, are increasingly being reported as the cause of disease in fish, (Schmidtke & Carson, 1994).

In the past, the term "streptococcosis" has been coined for fish disease caused by lactic acid bacteria. Also, most of the literature reviewed in this thesis commonly used

the term despite the fact that is now believed, after greatly advanced techniques in the methodology of the identification of these organisms, that these disease-producing organisms could be streptococci, enterococci, lactococci or some other organism as yet not named. Kusuda & Salati (1993) suggested that the disease be renamed "enterococcal infection" instead of "streptococcal infection" or "streptococcicosis", names that had been previously used. "Streptococcosis" of fish should perhaps be regarded as a complex of similar diseases caused by different genera and species of Gram-positive cocci, most capable of producing similar symptoms of disease (Bercovier *et al.* 1997a).

In 1976 Kusuda *et al.* (1976) began to examine the phenotypic characteristics of a *Streptococcus* sp. isolated from an epizootic of cultured yellowtail (*Seriola quinqueradiata*). Their results placed the organism into a group of bacteria known then as *Streptococcus* group 111. This group of bacteria also included the species *S. faecalis, S. faecium* and *S. durans*. The organism was found to be different from any established species of the genus *Streptococcus* (Kusuda *et al.* 1976). Then, in 1991, when the phenotypic properties of streptococci isolated from diseased specimens of cultured yellowtails and eels (*Anguilla japonica*) were again examined, the isolates could be related to, but distinguished from, the other species of the recently revived genus *Enterococcus* sp. by several biochemical characteristics and by the absence Lancefield's group D antigen (Kusuda *et al.* 1991). The genotypic property, using DNA-DNA hybridisation determined by the thermal melting point temperature, showing a guanine-plus-cytosine (G+C) value of 44 mol % was sufficiently low to warrant distinguishing these isolates from reported *Enterococcus* sp. and the name *Enterococcus seriolicida* was proposed (Kusuda *et al.* 1991).

Since then, it has been suggested that the major causative agent of "streptococcosis" in fish is in fact *Lactococcus garvieae* (Eldar *et al.* 1996; Doménech *et al.* 1996; Teixeira *et al*, 1996) an organism which has had a chequered taxonomic history. The organism classified as *Streptococcus garvieae* by Collins *et al.* (1983) was first described as a novel species by the British microbiologist Ellen Garvie in 1981. She

**Table 1.5 Characteristics of type strains of *Lactococcus* sp. and *Enterococcus* sp. (Teixeira et al, 1996)**

| | *Lactococcus garvieae* ATCC 43921 | *Lactococcus garvieae* SS 1290 | *Enterococcus seriolicida* ATCC 49156 | *Lactococcus lactis* ATCC19435 | *Enterococcus durans* ATCC 11576 | *Enterococcus durans* ATCC 11576 | *Enterococcus hirae* ATCC 8043 |
|---|---|---|---|---|---|---|---|
| Growth at: 10 °C | + | + | + | + | + | + | + |
| 45 °C | + | + | + | + | + | + | + |
| Growth in: 6.5%NaCl | + | + | + | - | + | + | + |
| Esculin hydrolysis | + | + | + | + | + | + | + |
| Arginine hydrolysis | + | + | + | + | + | + | + |
| Hippurate hydrolysis | + | + | + | + | - | + | + |
| VP reaction | + | + | + | + | + | + | + |
| PYR | + | + | + | - | + | + | + |
| Arabinose | - | - | - | - | - | - | - |
| Glycerol | - | - | - | - | + | - | - |
| Lactose | + | - | - | + | + | + | + |
| Mannitol | + | + | + | - | - | - | - |
| Melibiose | - | - | - | - | - | - | - |
| Raffinose | - | - | - | - | - | - | + |
| Ribose | + | + | + | + | + | + | + |
| Sorbitol | - | - | - | - | - | - | - |
| Sucrose | - | - | - | - | - | - | + |
| Trehalose | - | - | - | - | - | - | + |
| Lancefield group | - | - | - | N | D | D | D |

performed biochemical and chemotaxonomical studies on a *Streptococcus* sp. isolated from a case of bovine mastitis and which appeared to be closely related to *Streptococcus lactis* (Garvie *et al.* 1981; Collins *et al.* 1983). This organism has since been reclassified as *Lactococcus garvieae* (Schleifer *et al.* 1985; Elliott *et al.* 1991; Eldar *et al.* 1996; Doménech *et al.* 1996). *Lactococcus garvieae* has also been isolated from subclinical mastitis in water buffalo (Teixeira *et al.* 1996), bovine milk, horse and cat faeces and turtle conjunctiva (Pot *et al.* 1996).

The new type strain, *Enterococcus seriolicida,* as proposed by Kusuda *et al* (1993) has since been refuted by Teixeira *et al* (1996) in an article which probes the taxonomy of the infectious agent of mastitis in water buffalo. Phenotypic tests (see ` 1.5) and whole-cell protein profiles suggest that *Enterococcus seriolicida* is closely related to the organism associated with bovine mastitis, *Lactococcus garvieae* (Teixeira *et al,* 1996). The type strain (ATCC 49156) of *Enterococcus seriolicida* has also been shown by M. D. Collins (unpublished) to be genotypically identical to *Lactococcus garvieae* (100% 16S rRNA similarity) (Devriese *et al.* 1993). Therefore, since *Lactococcus garvieae* was described first, *Lactococcus garvieae* is a senior subjective synonym of *Enterococcus seriolicida* and, hence, the name *Lactococcus garvieae* should be retained. Strain ATCC 49156 should be renamed *Lactococcus garvieae,* and the type strain of *Lactococcus garvieae* remain ATCC 43921 (= NCDO 2155) (Teixeira, 1996). It has now become obvious that, although there may be organisms similar to streptococci causing "streptococcosis" disease in fish, the predominant organism appears to be *Lactococcus garvieae.* Using DNA-DNA hybridization studies Ghittino *et al.* (1997) confirmed that *Enterococcus seriolicida* was identical to *Lactococcus garvieae.* They compared strains causing streptococcosis from four different continents (Africa, Asia, Australia and Europe) using biochemical and genetic methods. DNA-DNA hybridization showed that all strains except one belonged to the same genospecies, *Lactococcus garvieae.*

### 1.2.2 Reports of "streptococcosis" in fish worldwide

Diseases caused by catalase-negative Gram-positive cocci have traditionally been designated streptococcosis and are major diseases of fish species worldwide. These diseases have been reported from many parts of the world including the Far East

**Table 1.6 Summary of the occurrence of "streptococcosis" in fish worldwide**

| COUNTRY | SPECIES | HOST | AUTHOR |
|---|---|---|---|
| Japan | *Streptococcus* sp. | Yellowtail | Kusuda *et al.* 1976 |
| | *Streptococcus* sp. | Eel | Kusuda *et al.* 1978 |
| | *Streptococcus* sp. | Yellowtail | Kitao *et al.* 1979 |
| | *Enterococcus seriolicida* | Yellowtail & eels | Kusuda *et al.* 1991 |
| Singapore | *Streptococcus* sp. | Siganids | Foo *et al.* 1985 |
| Korea | *Streptococcus* sp. | Yellowtail | Park *et al.* 1987 |
| | *Streptococcus* sp. | Flounder | Kim & Lee, 1994 |
| South Africa | *? S. faecalis* | Rainbow trout | Roode, 1977 |
| | *Streptococcus* sp. | Rainbow trout | Boomker *et al.* 1979 |
| | *Streptococcus* sp. | Rainbow trout | Barham *et al.* 1979 |
| | *Streptococcus* sp. | Rainbow trout | Bragg & Broere, 1986 |
| Australia | *Streptococcus* sp. | Rainbow trout | Munday & Copland, 1981 |
| | ? *Enterococcus* sp. | Rainbow trout | Carson & Statham, 1993 |
| | *Streptococcus* sp. | Rainbow trout | Munday *et al.* 1993 |
| U.S.A. | *Streptococcus* sp. | Golden shiner | Robinson & Meyer, 1966 |
| | *Streptococcus* sp. | Various species | Baya *et al.* 1990 |
| | *S. iniae* | Striped bass | Stoffregen *et al.* 1996 |
| | *S. iniae* | Tilapia | Perera & Johnson, 1994 |
| | *S. iniae* | Tilapia | Perera *et al.* 1997 |
| Italy | *Streptococcus* sp. | Rainbow trout | Ceschia *et al.* 1992 |
| | *Streptococcus* sp. | Rainbow trout | Ghittino & Prearo, 1992 |
| | *Enterococcus*-like | Sturgeon | Salati *et al.* 1996 |
| Spain | *L. garvieae* | Rainbow trout | Doménech *et al.* 1993 |
| | ? *Enterococcus* sp. | Turbot | Toranzo *et al.* 1994a |
| | *S. parauberis* | Turbot | Doménech *et al.* 1996 |
| Israel | *S. shiloi* & *S. difficile* | Tilapia & rainbow trout | Eldar *et al.* 1995a |
| | *S. iniae* | Rainbow trout | Eldar *et al.* 1995b |
| | *L. garvieae* | Rainbow trout | Eldar *et al.* 1996 |
| | *L. garvieae* | Rainbow trout | Eldar *et al.* 1996 |
| | *Streptococcus* sp. | Tilapia | Bunch & Bejerano 1997 |
| France | *V. salmoninarum* | Rainbow trout | Michel *et al.* 1997 |

(Japan, Taiwan and Korea), USA, South Africa, Australia, Israel and Europe (Spain, Italy, and France) (Bragg *et al.* 1989a, Carson & Statham, 1993, Ghittino *et al.* 1996; Plumb, 1994; Doménech *et al.* 1996; Michel *et al.* 1997). In both Japan and South Africa streptococcosis is now considered to be one of the major diseases affecting key farmed species of fish and accounts for a significant proportion of fish lost to bacterial disease (Carson & Munday, 1990). The host range of the organisms, be they *Streptococcus, Enterococcus or Lactococcus,* is not extensive but does include a number of commercially important species such as rainbow trout (*Oncorhynchus mykiss*), tilapia (*Tilapia nilotica*) yellowtail, turbot (*Scophthalmus maximus*), eels (*Anguilla anguilla*), carp (*Cyprinus carpio*), golden shiner (*Notemigonus crysoleucas*) and ayu (*Plecoglossus altevelis*) (Carson & Munday, 1990). The occurrence of "streptococcal" disease in fish is summarised in Table 1.6

Various aspects, including the characteristics of streptococcosis, the isolation of the pathogen and characterisation of the isolates, were reviewed by Austin & Austin in 1993. It was found that substantial biochemical diversity existed between *Streptococcus* species isolated from different species of fish from different geographical distributions (Bragg *et al.* 1989a).

### 1.2.2.1 South Africa

Boomker *et al.* (1979) documented the first outbreak of streptococcosis in South Africa which occurred in the Transvaal in 1974, during which excessive mortalities were mostly seen in the bigger rainbow trout. A faecal streptococcus belonging to the Lancefield group D, growing at 45°C but not at 10°C, was isolated from the spleen, liver and kidneys of affected fish (Boomker *et al.* 1979). Although the symptoms were similar to "lactococcosis", it is not likely to be *Lactococcus garvieae* because of the presence of the group D antigen.

Bragg & Broere (1986) isolated a similar organism, from the same geographic area, from fish displaying typical symptoms of streptococcosis. Unlike Boomker's isolate this organism grew at 10°C, but not at 45°C and had similar phenotypic characteristics to the organism now thought to be *Lactococcus garvieae* (see Table 1.7).

**Table 1.6** Physiological and biochemical characteristics of streptococci associated with fish disease

| | Foo *et al* 1985 | Bragg & Broere 1986 | Baya *et al* 1990 | Kusuda *et al* 1991 | Ceschia *et al* 1992 | Carson *et al* 1993 | Toranzo *et al* 1994a | Kim & Lee 1994 | Teixera *et al* 1996 | Domenech *et al* 1996 |
|---|---|---|---|---|---|---|---|---|---|---|
| Growth at: | | | | | | | | | | |
| 10°C | + | + | | | | + | + | - | + | + |
| 22°C | | | + | | + | | | | | + |
| 37°C | | | | | + | + | + | + | | + |
| 45°C | - | | - | + | | - | - | weak | + | - |
| pH 9.6 | + | | + | | | | weak | weak | | - |
| Growth in: | | | | | | | | | | |
| 4.5% NaCl | | | | | | | | | | + |
| 6.5% NaCl | + | | | + | | + | + | weak | + | - |
| 0.01% TTZ | | | | + | | | | | | |
| Esculin | + | + | | | + | + | + | | + | |
| Haemolysis | α | | | α | | | α | α | | |
| Arginine hydrolysis | | + | + | + | + | + | + | | + | + |
| Hippurate hydrolysis | - | - | | - | | - | + | | - | +/- |
| VP/ acetoin reaction | | | + | + | + | + | + | - | + | + |
| PYR | + | | | | + | + | + | | + | + |
| Arabinose | - | - | - | | - | - | - | - | - | - |
| Glycerol | + | | | - | | - | - | | - | - |
| Lactose | - | - | | - | - | - | +/- | - | + | + |
| Mannitol | + | - | - | + | + | + | + | | - | + |
| Melibiose | - | | | - | | - | | | - | - |
| Raffinose | - | - | | - | - | - | - | | - | - |
| Ribose | + | | | + | | | + | | + | + |
| Salicin | + | + | - | | | + | | - | | + |
| Sorbitol | - | - | - | + | - | - | +/- | | - | + |
| Starch | | - | | | + | - | - | | | - |
| Sucrose | + | | + | - | | - | | - | - | |
| Trehalose | + | + | - | | + | + | + | | + | + |
| Lancefield group | | | B | Not D | | Not D | No group | | No group | |

### 1.2.2.2 Japan

Minami (1979) isolated a bacterium similar to *Streptococcus faecalis* and *Streptococcus faecium* from a streptococcal infection in cultured yellowtail, and found that the potential source of the infection was the feed. When fresh and frozen fish used for feed were examined, the bacterium was isolated from several of the feeds and was found to remain alive for over 6 months at low temperatures, especially frozen.

In 1976, Minami *et al.* (1979) isolated streptococci different to the bacteria previously isolated from cultured yellowtails by Kusuda *et al.* (1976) and which had been described by them as being similar to *Streptococcus faecalis* and *Streptococcus faecium.* Two isolates were found to be a β-haemolytic streptococcus, with similar characteristics to that of *S. equisimilis.* The strain caused typical symptoms of streptococcosis including corneal opacity and pericarditis in pathogenicity tests and, therefore, maybe should be considered as part of the earlier epizootic.

In Japan in 1974 a new pathogenic bacterium identified as belonging to the genus *Streptococcus* was isolated from an epizootic of cultured yellowtail (Kusuda *et al.* 1976; Kusuda & Salati, 1993). It was initially characterised as *Enterococcus seriolicida* (Kusuda, 1993), but more recently re-characterised as *Lactococcus garvieae* (Doménech *et al.* 1993; Eldar *et al.* 1996; Teixeira *et al.* 1996). This organism is also thought to cause disease in cultured eel (*Anguilla japonica*), ayu (*Plecoglossus altivelis*), sea bream (*Pagrus major*), saurel (*Trochurus japonicus*) and flounder (*Paralichthys olivoceus*) (Kusuda & Salati, 1993)

### 1.2.2.3 The Far East (excluding Japan)

When mass mortality of cultured rabbitfish (*Siganus canaliculatus*) occurred in floating fish farms located off the north-east coast of Singapore, the bacteria isolated from the diseased fish were found to be organisms with characteristics similar to *Streptococcus faecalis* and *Streptococcus faecium* (Foo *et al.* 1985). Grouper (*Epinephelus tauvina*) and sea bass (*Lates calcarifer*) being cultured at the same time under the same conditions did not suffer mass mortality, suggesting that the rabbitfish may be more susceptible to certain streptococcal infections (Foo *et al.* 1985).

Mass mortality of flounder *Paralichthys olivaceous*, especially in fish weighing 6-10g occurred in Korea. (Kim & Lee, 1994). A *Streptococcus* strain, the characteristics of

which are given in Table 1.7, was isolated. The organism did not grow at 10°C and grew poorly at 45°C, was VP negative and the authors also reported that it was catalase positive which seems unlikely to be correct if the organism is a *Streptococcus* sp., let alone *Lactococcus garvieae*.

### 1.2.2.4 Spain

Since 1993, one of the most threatening diseases for juvenile and adult turbot cultured in Galicia (North West Spain) has been streptococcosis caused by an *Enterococcus*-like bacterium that causes high morbidity and serious economic loss (Toranzo *et al.* 1994a & b).

### 1.2.2.5 Australia

The first recorded outbreak of streptococcal disease in Australia occurred in salmonids in Tasmania in the late 1970s (B. L. Munday 1997 pers. comm.). It was found that there were differences in the characteristics between the streptococcal isolate causing peritonitis to the one causing septicaemia in trout (Munday & Copland, 1981). The *Streptococcus* sp. (probably *Carnobacterium* or *Vagococcus*) isolated from the cases of peritonitis, was not the same as the *Streptococcus* sp. isolated previously from a case of septicaemia on the same farm (see Table 1.8) (Munday & Copland, 1981).

More recently, in the late 1980s, the incidence of streptococcosis in Tasmania has escalated, with losses as high as 30 % in summer when water temperatures exceeded 18°C and up to 60 % in sea-run fish (Carson & Munday, 1990). The organism involved has been different from those described by Munday & Copland (1981) and will be described in detail in this thesis. Carson (1990) described one of the causes of the disease streptococcosis in Australia as *Streptococcus* sp. biovar 1 that he had isolated from sites including the kidney, the brain and the eye orbit. Since then it has been described as an *Enterococcus*-like bacterium (Carson *et al.* 1993) but is now believed to be, in fact, *Lactococcus garvieae*.

Although Tasmania was the major state in Australia for the incidence of streptococcosis, disease has occurred in other parts of Australia (Munday & Copland, 1981). New South Wales Fisheries had several outbreaks of streptococcosis in

**Table 1.8 Characteristics of two streptococcal isolates from farmed tablefish in Australia (Munday & Copland, 1981)**

| Reaction | *Streptococcus* sp. from peritonitis in trout | *Streptococcus* sp. from septicaemia in trout |
|---|---|---|
| Motility | - | - |
| Oxidase | - | - |
| Catalase from BA † | + | - |
| Catalase from NA ‡ | - | |
| Glucose | a | a |
| Mannitol | - | a |
| Salicin | a | - |
| Trehalose | a | a |
| Maltose | | a |
| Raffinose | - | - |
| Sorbitol | - | a |
| Arabinose | | a |
| Gelatin | - | - |
| Nitrate | - | |
| Litmus milk | No change | RAC |
| Citrate | - | |
| Urea | - | |
| Growth at 37°C | - | |
| Haemolysis | α | α |
| Esculin | + | + |

† Blood agar

‡ Nutrient agar

snapper at the hatchery at the Port Stephens Research Centre in 1993 and possibly 1994 (S. Battaglene, 1997 pers. comm). The disease occurred in juvenile snapper cultured at high density, and when they had about 25mm in length. The problem was solved by treating with antibiotics and then in the long term, by improved husbandry (lower density and better food). Also although *Vibrio splendidus* was the dominant organism isolated from sea cages in Botany Bay, Sydney, some *Streptococcus* sp. were isolated from on-grown snapper (*Pagrus auratus*) (S. Battaglene, 1997 pers. comm.; S. Fielder, 1997 pers. comm.).

Streptococcosis also occurs in Queensland in areas around Townsville, with barramundi (*Lates calcarifer*) being the fish affected. The first occurrence was from 1992 to 1994, and the causative organism was identified as *Streptococcus iniae*. Since then there has been an ongoing problem with fish held in sea cages. Rainfall seems to be a contributing factor as most of the outbreaks have occurred after heavy rainfall as

this lowers the salinity and increases the amount of sediment in the water (I. Anderson, 1997 pers. comm.; A. Thomas, 1997 pers. comm.; E. Bromage, 1997 pers. comm.).

The disease causes losses of up to 80% in sea-caged fish, and most of the year round it was present in the water and there was asymptomatic carriage of the organism in the fish of up to 12%. Warmer water temperatures of up to 36°C during summer caused the disease to disappear from the fish and the environment. This is thought to be due to the fact that the bacteria has an optimum growth temperature of 24°C and will not grow at 37°C. The organism has found to be more pathogenic in the lower the salinity and is highly pathogenic in fish held in fresh water (E. Bromage, 1997 pers. comm.).

There have been outbreaks of streptococcosis in Victoria but there is no more information available other than that they occurred between 1983 and 1987 and the organisms are the same strains as the Tasmanian strains of what is now believed to be *Lactococcus garvieae*.

#### 1.2.2.6 United States of America

Baya *et al.* (1990) found that continuing mortality of bluefish (*Pomatomus saltatrix*), striped bass (*Morone saxatilis*) and sea trout (*Cynoscion regalis*), observed around Chesapeake Bay, Maryland, USA was caused by a non-haemolytic Group B *Streptococcus* species. Therefore, it is unlikely to be the same organism that is now known as *Lactococcus garvieae*. Chesapeake Bay is a badly polluted waterway and the resultant effects on the fish immune system may have influenced the course of the disease.

#### 1.2.2.7 Israel

In Israel, streptococcal disease affecting tilapia (*Oreochromis aura* x *Oreochromis nilotica* hybrids) and rainbow trout was found to be caused by two *Streptococcus* species, *Streptococcus shiloi* and *Streptococcus difficile*, with mortality ranging from 30% in tilapia to 50% in the trout (Eldar *et al.* 1995a). Eldar *et al.* (1995b) have performed further work on the species *Streptococcus shiloi* and it has now been

shown that the fish-pathogenic organism, *Streptococcus iniae*, is phenotypically and genotypically the same as *Streptococcus shiloi* and therefore *Streptococcus shiloi* should be considered a junior synonym for *Streptococcus iniae*. Bunch & Bejerano (1997) isolated *Streptococcus* spp. from diseased and healthy tilapia hybrids, healthy carp (*Ciprinus carpio*), diseased mullet (*Mugil cephalus*) and striped hybrid bass (*Morone saxatilis* x *Morone chrysops*). These were divided into two groups, according to the fermentation or non-fermentation of mannitol, and the presence of either α or γ haemolysis. These isolates were then tested using two commercial multi-test systems (API-50CH and rapid ID 32 STREP) and the results tabulated, but, surprisingly, no identification was documented. By extracting the results of the rapid ID 32 STREP it is unlikely that either of these organisms were the same as *Lactococcus garvieae* and, from the results given by the rapid ID 32 STREP, they are most likely to be as yet undescribed species.

**1.2.2.8 Italy**

Ghittino & Prearo (1992) reported cases of streptococcosis of rainbow trout in Northern Italy during the summer and autumn of 1991. The strains of streptococci isolated were found to belong to the Lancefield group D and were identified as *Streptococcus faecalis* and *Streptococcus faecium,* which are now classified as *Enterococcus faecalis* and *Enterococcus faecium.* Ceschia *et al.* (1992) also reported outbreaks of streptococcosis during the same period as Ghittino & Prearo (1992) and tentatively identified the aetiological agent as a *Streptococcus* similar to the species *lactis* and different to the organism isolated by Ghittino & Prearo (1992).

Salati *et al* (1996) isolated an *Enterococcus*-like bacterium from farmed Adriatic sturgeon (*Acipenser naccarii*) in Northern Italy. This study included comparison of the Italian isolate with the fish-pathogenic bacterium *Enterococcus seriolicida* (ATCC 49156) and two other strains of *Enterococcus seriolicida* (E-092 and E-014) using conventional methods and the commercial system API-20S and Vitek automated system (both bioMérieux, France). Salati *et al* (1996) found that their isolate was similar to but not the same as, the three type strains of *Enterococcus seriolicida.* Furthermore, the API 20S system identified all 4 strains as *L. lactis* and the Vitek system identified them as *E. faecium*, but this is probably due to the fact that these

systems are not designed or programmed to identify the species *Enterococcus seriolicida* or *Lactococcus garvieae* (bioMérieux Vitek, Australia, 1997 pers. comm.).

### 1.2.3 Predisposing conditions

Conditions that could contribute towards streptococcosis include poor water quality (Kim & Lee,1994) such as run off from farming practices in the area. This can contaminate the water with nitrogenous compounds and phosphates (Ceschia *et al.* 1992; Ghittino & Prearo, 1992). Also, the presence of pollutants contributed by industry and sewage in areas such as Chesapeake Bay and it's tributaries can contribute to increased incidence of fish disease (Baya *et al.* 1992). Stress resulting from overcrowding (Munday & Copland, 1981), lack of oxygen, poor feed quality and constant handling can cause latent disease to manifest itself (Roode, 1977; Purser, 1993). Warmer water temperature (≥18 °C) often appears to be closely related to the occurrence of the disease in farmed rainbow trout (Carson & Munday,1990; Ghittino & Prearo, 1992; Ceschia *et al.* 1992; Munday *et al.* 1993; Munday, 1996), barramundi (E. Bromage, 1997 pers. comm.) and tilapia (Perera *et al.* 1997; Bunch & Bejerano 1997).

Intensified feeding of tilapia in Israel during summer resulted in increased oxygen consumption and increased ammonia, nitrate and nitrite concentrations in the water, which, with increased water temperature, promoted the spread of disease, increasing morbidity (Eldar *et al.* 1995a). Bunch & Bejerano (1997) experimented with the effects of low dissolved oxygen and high nitrate concentrations on the susceptibility of hybrid tilapia to streptococcal infection. Both stress factors increased the mortality, but no additive effect was seen when both these factors were applied simultaneously. Extreme stress, especially that experienced by the fish when they are acclimated from fresh water to sea water, has been known to cause severe outbreaks of infection (Carson & Munday, 1990; Garland & Carson, 1987).

### 1.2.4 Signs of disease

Typical external symptoms of streptococcosis are bilateral exophthalmos (Nieto *et al.* 1995; Eldar *et al.* 1995a; Chang & Plumb,1996), congestion of the pectoral and

caudal fins and petechiae on the inside of the opercula (Kusuda *et al.* 1976; Baya *et al.*1992; Kusuda & Salati, 1993; Doménech *et al.* 1993). Chronic infection may also occur and a prominent feature of this condition is the pronounced bilateral exophthalmia or "pop-eye" (Figure 1.1) that frequently leads to complete degeneration of the eye. (Boomker *et al.* 1979; Carson & Munday, 1990; Ceschia *et al.*1992; Ghittino & Prearo, 1992; Toranzo *et al.* 1994a; Kim & Lee, 1994; Eldar *et al.* 1995a).

Clinical signs in moribund fish infected with "streptococci" generally include darkly pigmented skin, anorexia and exhibition of a lethargic, erratic, spiralling swimming pattern with a curved body (Figure 1.2) and survivors may develop a scoliosis of the spine (Figure 1.3) (Carson & Munday, 1990; Baya *et al.* 1990; Doménech *et al.* 1993; Plumb, 1994; Toranzo *et al* 1994a; Nieto *et al.* 1995; Eldar *et al.* 1995a; Chang & Plumb, 1996).

Infection of fish with streptococci usually produces a typical septicaemia, seen as an overwhelming infection with invasion of the bloodstream (Carson & Munday, 1990; Ceschia *et al.* 1992). Internally, there is marked congestion, the spleen may be enlarged and the intestine haemorrhagic and filled with a yellowish fluid (Nieto *et al.* 1995). The viscera may also be covered with a purulent white layer (Bragg & Broere, 1986; Baya *et al.* 1990; Doménech *et al.* 1993; Toranzo *et al.* 1994a).

Trout infected by *Streptococcus iniae* and tilapines infected by *Streptococcus difficile* mainly exhibit panophthalmitis (pop eye) and meningitis/meningoencephalitis is the only other finding. *Lactococcus garvieae*-infected trout usually develop a systemic, hyperacute infection with scattered haemorrhages; lesions are encountered not only in the eye and brain, but also in viscera such as the intestine (erosive enteritis with pseudomembranes), heart (pericarditis) and liver (peliosis hepatis) (Ghittino *et al.* 1996). Usually the organisms appear to have a marked neurotropism and may be

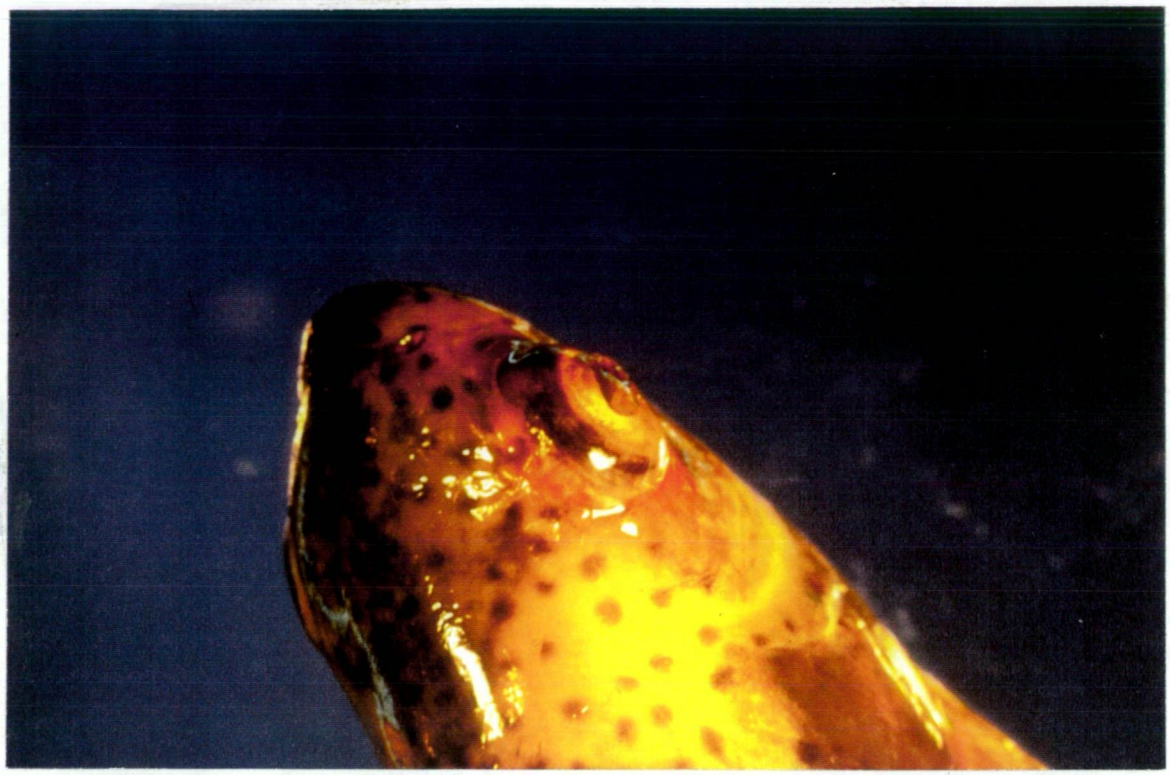


**Figure 1.1** Rainbow trout displaying rupture of the eye and exophthalmia or "pop-eye"

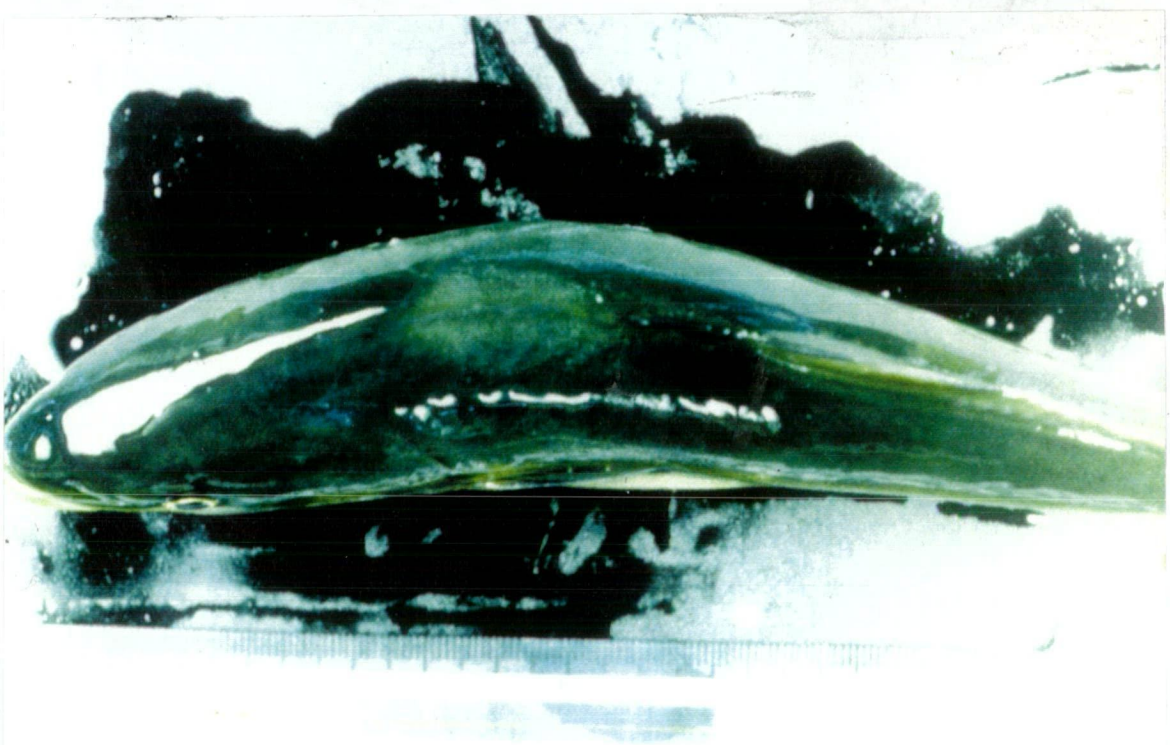


**Figure 1.2** Typical curving of the body and darkening of skin

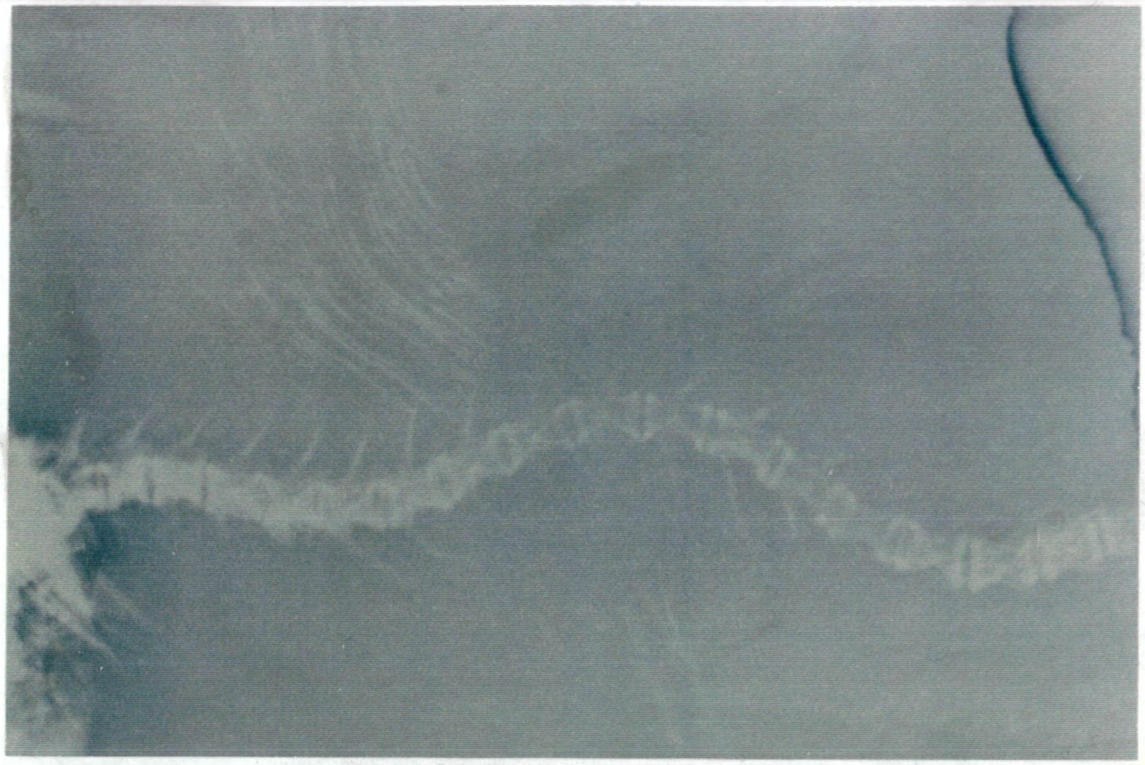


**Figure 1.3** X-ray showing scoliosis of the spine


**Figure 1.4** Dorsal view of the brain showing haemorrhage **A** and swelling **B**

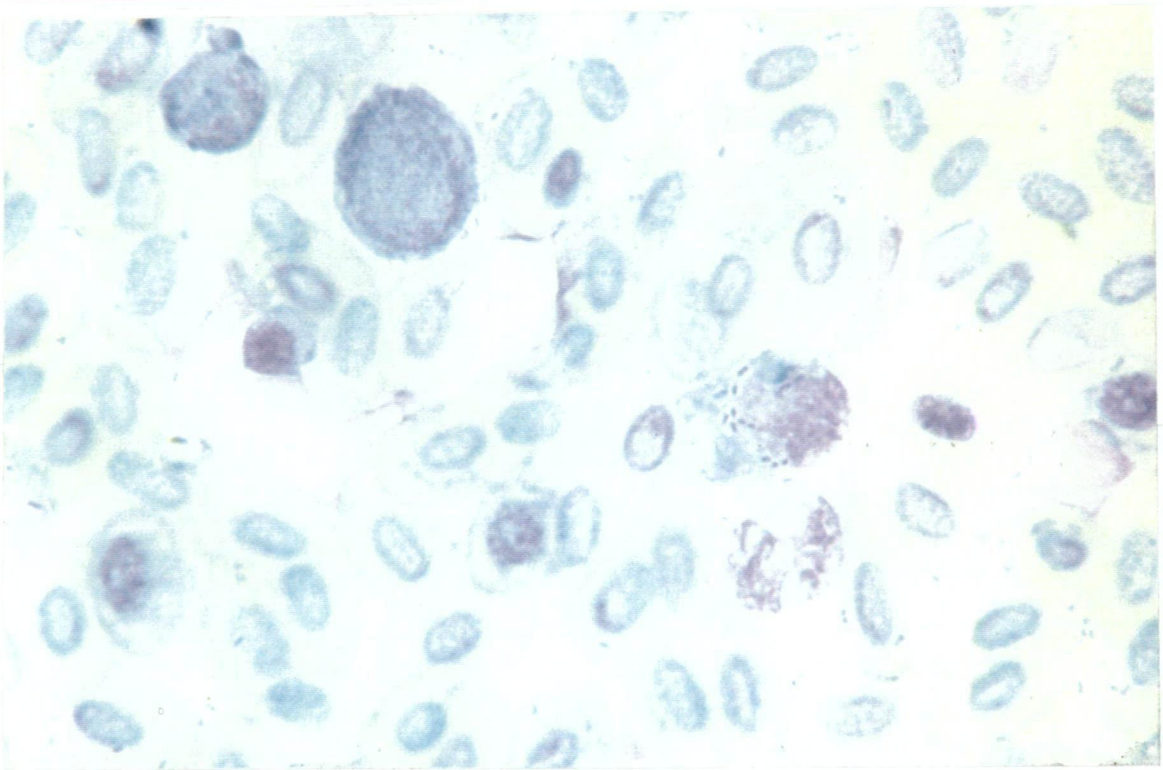


**Figure 1.5** Macrophages in the spleen engulfing the Gram-positive bacteria

readily isolated from the brain which may be haemorrhage (Figure 1.4). Because of the typical signs, it is easy to presumptively identify an enterococcal infection, however, the diagnosis must be confirmed microbiologically (Baya *et al* 1990).

### 1.2.5 Histopathology of diseased fish

Histopathology of tilapia and channel catfish experimentally infected with *Streptococcus* sp. revealed: meningitis; polyserositis of the heart, liver, spleen, ovary and kidney; splenitis; ovaritis; and myocarditis, whilst eyes showed inflammation in the choroid region (Chang & Plumb, 1996). Bacteria were seen attached to the erythrocytes of the hepatic vein and engulfed by macrophages in the spleen (Figure 1.5), heart, ovary and meninges in tilapia infected with *Streptococcus* sp. (Chang & Plumb, 1996). Macrophages containing Gram-positive cocci are found in the tissue,

especially spleen, kidney, heart and brain, on histological examination (Munday, 1996).

Turbot infected with an enterococcus-like organism showed muscle haemorrhages, acute branchitis, and suppurative inflammation of periorbital tissues, eyeball, meninges and brain with extensive haemorrhages, ulceration and purulent inflammation of the skin and necrosis of spleen and kidney with fatty changes in the liver (Nieto *et al.* 1995).

### 1.2.6 Methods for isolating and identifying the causative organisms

#### 1.2.6.1 Physiological features

The causative organism of the disease "streptococcosis" in yellowtail in Japan was a Gram-positive coccus with non-sporing ovoid cells occurring in short chains and growing at 10°C to 45°C with the optimum temperature appearing to be between 20°C and 37°C (Kusuda & Salati, 1993). Growth also occurred at a concentration range of 0-6.5% salt and pH about 7.5. Although the organism causing the "streptococcosis" showed no detectable Group D antigen, it showed many biochemical and physiological characteristics found in the genus *Enterococcus.* (Kusuda *et al.* 1976; Kusuda & Salati, 1993).

In samples taken from diseased striped bass *Morone saxatilis* (Walbaum), bluefish *Pomatomus saltatrix* and sea trout *Cynoscion regalis,* Gram stains of smears made from kidney, spleen, liver and brain tissues showed large numbers of streptococcus-like organisms, and a non-haemolytic Gram-positive coccus, occurring singly, in pairs or in chains, was isolated in pure culture (Baya *et al.* 1990). The biochemical characteristics of this isolate is shown in Table 1.7 (P12) along with other streptococci associated with fish disease.

Of the enterococci, the classical well-known species *Enterococcus faecalis* and *Enterococcus faecium,* on which the genus description (Schleifer and Kilpper-Bälz 1984) was based, have a number of characters in common which distinguish them from other catalase-negative, Gram-positive, facultatively-anaerobic cocci. Their ability to grow both at 10°C and 45°C, in 6.5% NaCl broth and at pH 9.6 and most

importantly their characteristic to usually possess the Lancefield group D antigen. However, *E. cecorum, E. columbae, E. dispar, E. saccharolyticus* and many strains of the *avium* species group and even *E. durans* fail to react with Lancefield group D antisera (Devriese *et al.* 1993).

In 1983, it was thought that only three species of streptococci (*Streptococcus lactis*, *Streptococcus cremoris* and *Streptococcus raffinolactis*) could grow at 10°C but not at 45°C, and that all three species contained the serological group N antigen (Collins *et al.* 1983). Since then these organisms have been reclassified as *Lactococcus* sp. which are facultatively anaerobic or aerotolerant, grow at 10°C but, unlike *Enterococcus* spp., not at 45°C (Schleifer & Kilpper-Bälz, 1987).

### 1.2.6.2 Basic identification techniques

Rapid, cost-effective methods for differentiation between *Streptococcus* sp. and *Enterococcus* sp. use a series of physiological tests including Lancefield serological grouping, pyrrolidonylarylamidase (PYR) (Bosley *et al.* 1983; Facklam & Collins, 1989), β esculin, hippurate, and β-D-glucosidase (βGUR). The enterococci show positive PYR and esculin tests (Kirby & Ruoff, 1995). Conventional biochemical techniques were used by Facklam & Collins (1989) to develop a system to identify *Enterococcus* sp. These tests included Gram stain, resistance to 30 µg vancomycin discs, pyrrolidonylarylamidase activity, gas production from glucose, bile esculin, 6.5% NaCl and 0.4% tellurite tolerance, reduction of 0.1% tetrazolium, hippurate hydrolysis and carbohydrate fermentation. As a result of this study, two new *Enterococcus* species were discovered (*Enterococcus raffinosus* and *Enterococcus solitarius*) and an identification system was designed to enable microbiologists to accurately identify most *Enterococcus* species, with a minimal number of conventional physiological tests (Facklam & Collins, 1989).

There was a high degree of homogeneity between the Australian and South African isolates of (*Enterococcus*-like) *Streptococcus* sp. as demonstrated by their whole cell protein profiles, although biochemical testing showed that, whereas the Australian strains produced acetoin and acid from mannitol or maltose, the South African isolates did not. (Carson *et al.* 1993).

The antibiograms of some of the lactococci were useful in discriminating between some species, since the antimicrobial susceptibilities of *Lactococcus lactis* and *Lactococcus garvieae* showed that *Lactococcus lactis* was susceptible to clindamycin but *Lactococcus garvieae* was resistant (Elliott & Facklam, 1996).

### 1.2.6.3 Commercial identification systems

Multi-test systems such as API ZYM have been used to identify bacterial fish pathogens including the Gram-positive *Enterococcus seriolicida,* β-haemolytic *Streptococcus* sp. and *Renibacterium salmoninarum* as well as Gram-negative bacteria including *Aeromonas salmonicida* and *Vibrio anguilllarum* (Sakai *et al.* 1993).The API 20S system was a useful tool when Gram positive cocci (mainly streptococci, enterococci and lactococci) could not be identified by conventional (i.e. not multi-test) methods (Facklam *et al.* 1985).

Shotts & Meads (1997) performed biochemical analysis of *Streptococcus* sp. isolated from fish. The isolates were tested by conventional, kit and automated methods resulting in the suggestion that many of these organisms were often misidentified due to the inadequate databases for their identification.

### 1.2.6.4 Immunological techniques

It appears that fish immunoglobulin is represented by only one class, corresponding to that of mammalian IgM or macroglobulin (Gudkovs, 1988; Pilström & Bengtén, 1996). In fish, immunoglobulins are found in most tissues and fluids including plasma, lymph, skin, gill and gut mucus and bile. They are also found in association with the membranes of the lymphocytes where they act as antigen-specific receptors. Often the high permeability of the blood vascular system of fish is responsible for the immunoglobulin being present in these tissues (Gudkovs, 1988).

Diagnosing disease often depends on the ability to detect proteins on the surface of the micro-organisms responsible, proteins which are specific for that organism and often are present only in small amounts in the clinical samples. The distinctive character of individual proteins is conveniently revealed by using antibodies, and

enzyme-linked immunosorbent assay (ELISA) is one of the more commonly used techniques for this (Eaton, 1994).

In yellowtail naturally infected with *"Streptococcus"* sp., antibody production in the serum and in the intestinal mucus was studied by agglutination using cellular antigen, and passive haemagglutination using endotoxin and exotoxin of the *Streptococcus* species (Kusuda & Takagi, 1983). The results of that study showed that yellowtail produces antibody against antigens of *Streptococcus* sp., but it is insufficient to be protective, therefore, there is a greater possibility of an outbreak of infection in these yearling and adult fish (Kusuda & Takagi, 1983).

Antiserum raised in rabbits against the streptococcal organism causing mortality in fish, including bluefish, striped bass and sea trout, in Chesapeake Bay was used in agglutination tests to compare the homology of this organism to other organisms causing streptococcosis in fish in the USA (Baya *et al.* 1990). The antisera reacted with the organism isolated by Plumb *et al* during the summer of 1972 along the Alabama and Florida coasts. However, isolates of the bacteria sent to Dr Tadatoshi Kitao at Miyazaki University, Japan did not agglutinate with the antisera prepared against *"Streptococcus"* sp. that cause disease in cultured yellowtail or freshwater fish in Japan, suggesting that the organism is not the same as that now thought to be *Lactococcus garvieae* (Baya *et al.* 1990).

Bragg, (1988) used an indirect fluorescent antibody technique for the rapid identification of streptococcosis in rainbow trout *Salmo gairdneri* (now termed *Oncorhynchus mykiss*). Antisera were raised in New Zealand white rabbits and the indirect fluorescent antibody tests (IFAT) was performed on pure cultures of bacteria, as well as kidney, spleen and liver smears from diseased and experimentally infected fish and proved to be a sensitive and rapid technique for the diagnosis of streptococcosis in rainbow trout.

Direct and indirect fluorescent antibody techniques were evaluated by Kusuda & Kawahara (1987) as methods for identification of four yellowtail pathogens including *"Streptococcus"* species. Antibody was obtained from rabbits immunised with formalin-killed bacterial cells from the cultures and the conjugate was celite-FITC

goat anti-rabbit IgG. Although both methods (direct and indirect) were positive, the results suggested that the sensitivity and specificity of the direct test was better than that of the indirect test.

### 1.2.6.5 Molecular biological techniques including RAPD-PCR

One of the most significant outcomes of the biotechnology revolution has been the development of techniques to isolate, purify and manipulate nucleic acids from organisms. The order in which the four nucleotides (A, C, T & G in the case of DNA) occur in a stretch of nucleic acid can now be determined rapidly.

The nucleic acid sequence is often characteristic of the organism from which it was derived and its uniqueness enables detection and identification of micro-organisms in clinical samples. There are two ways of doing this, by using gene probes or polymerase chain reaction (PCR) technology (Eaton, 1994).

A simple method for extracting large amounts of plasmid DNA from lactic acid bacteria uses glass beads to break cells, producing plasmid DNA pellets in less than one hour, has successfully been tested on various bacteria including *Lactococcus, Streptococcus & Enterococcus* species. The plasmid DNA produced therefore creates a technique for rapid screening of plasmid contents of wild and transformed Gram-positive bacteria (Frere, 1994).

The application of molecular approaches for the classification of Gram-positive cocci (DNA-DNA hybridisation studies coupled with 16S sequencing) has shown that at least five different species are pathogenic to fish: *Streptococcus iniae, Streptococcus difficile, Lactococcus garvieae, Lactococcus piscium, and Vagococcus salmoninarum.* Streptococcosis of fish should, therefore, be regarded as a complex of similar diseases caused by different genera of Gram-positive cocci, each capable of inducing CNS damages, along with various degrees of multisystem organ involvement (Ghittino *et al.* 1996).

In recent years, molecular techniques have moved rapidly from a few specialised laboratories into the mainstream of microbial systematics. Indeed molecular

sequences, particularly 16S rRNA, may one day displace DNA mol % G + C as required information for the description of a new species (Giovannoni, 1991).
A recent study on the Gram-positive organisms causing outbreaks of disease in turbot in Spain highlights the problem of relying on conventional methods of identification of the pathogen (Doménech *et al.* 1996). The enterococcus-like bacterium described by Toranzo *et al* in 1994, was found to be in fact *Streptococcus parauberis*, as determined by 16S rRNA sequencing (Doménech *et al.* 1996). Because of such discrepancies it is suggested that identification by molecular methods, such as partial rRNA gene sequencing , should be used whenever possible to confirm presumptive phenotypic identification of bacterial fish pathogens (Doménech *et al.* 1993).

It is history about the polymerase chain reaction now, but everyone interested in molecular biology knows that Kary Mullis "ran into the idea one night in the mountains of California" (Mullis, 1990). The principle of the PCR (polymerase chain reaction) is summarised in Figure 1.6. The first descriptions of the use of PCR using random amplifier primers was by Williams *et al*, 1990 and Welsh & McClelland, 1990. This general DNA typing approach has been used for bacteria including *Listeria* and *Escherichia coli* (Niederhauser *et al.* 1994). It provides an unequivocal means of differentiating *Vagococcus* sp. from related lactic acid bacteria such as *Enterococcus* & *Lactococcus* sp. (Williams & Collins, 1992). Further identification at the species level involves the use of synthetic oligonucleotide probes specific for vagococci which were designed from 16S rRNA sequence data and molecular hybridisations with PCR-amplified rDNA targets (Williams & Collins, 1992). PCR was also used for the identification of a number of clinical isolates of enterococci that could not be identified to species level by the API 50CH system (Dutka-Malen *et al.* 1995).

Random amplified polymorphic DNA (RAPD) profiling by PCR can be used to provide a simple and efficient comparison of clinical and environmental bacterial isolates (see Table 1.9) (Sandery *et al.* 1994; Mileham, 1995; Oakey *et al.* 1996). RAPD has an absolute requirement for a pure culture of the target organism, but requires no knowledge of the DNA sequence because the method depends on random

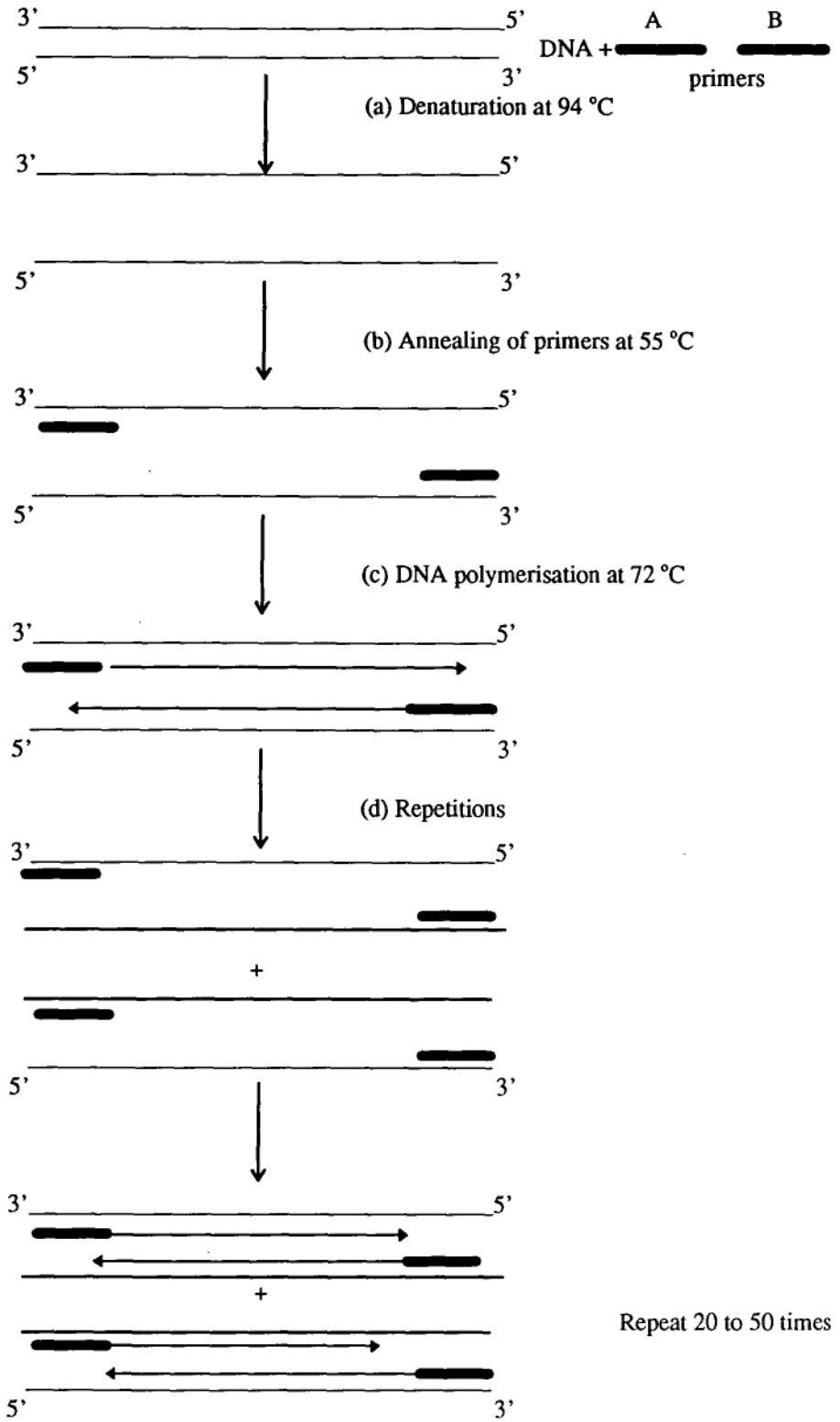



**Figure 1.6** Schematic representation of a typical PCR assay. First the target DNA is denatured by heat (a). Upon cooling, the primers which flank the desired segment of DNA, hybridise with complementary targets (b), and then the DNA synthesis takes place (c), starting from the primer. Theses steps are repeated (d) 20-50 times to obtain large quantities of the desired DNA fragment (Stansfield et al, 1996)

priming of the PCR using an oligonucleotide primer (or pair of primers) of arbitrary sequence. Normally, the fidelity of the hybridisation of an oligonucleotide primer to a denatured DNA template depends on many factors, including the length and the base composition of the primer. However, with RAPD, different primers produce different RAPD profiles with the same DNA sample. DNA samples from different members of a strain or species should all have identical and unique RAPD profiles provided that an appropriate primer is used, and these will differ from other strains or species. This has been successfully applied to a variety of organisms including bacteria, fungi, plants and animals (Mileham, 1995).

Random DNA sequences (usually 10 bases long) are used as primers to initiate the polymerase reaction. Each primer will generate a different set of sequences of amplified DNA from the target genomic DNA. The bands in this case are simply visualised by fluorescence in the presence of a dye after electrophoresis. There is no need for southern blot transfer or radioactive probes (Mileham, 1995).

**Table 1.9 Summary from the literature of the use of RAPD-PCR for the typing of bacteria (Sandery *et al.* 1994; Oakey *et al.* 1996; Miyata *et al.* 1996)**

| Organism | Year | Author |
|---|---|---|
| *Campylobacter* sp. | 1992 | Mazurier *et al* |
| *Listeria monocytogenes* | 1993 | McGowan *et al* |
| *Listeria* sp. | 1993 | Czajka *et al* |
| *Legionella pneumophilia* | 1994 | Sandery *et al* |
| Group A streptococci | 1994 | Seppala *et al* |
| *Vibrio* sp. | 1994 | Martin-Kearley *et al* |
| *Yersinia enterocolitica* | 1994 | Rasmussen *et al* |
| *Aeromonas* sp. | 1995 | Oakey *et al* |
| *Aeromonas salmonicida* | 1995 | Miyata *et al* |

Methods for identifying isolates of various pathogenic bacteria by DNA fingerprinting with random primers (RAPD), using many primers and selecting the primers that generate the most informative DNA pattern, have been described (Sakallah *et al.* 1995). A new method of introducing one or more degenerate nucleotides into the core rapid primer sequence at various nucleotide positions gives results comparable with using non-degenerate RAPD primers and expedites the need to generate unique DNA fingerprints of individual organisms (Sakallah *et al.* 1995).

The PCR program normally used for RAPD analysis is a typical PCR program except that the stringency during primer annealing is much lower than usual, because the primer used in the RAPD procedure is relatively short and has a arbitrary sequence. For random short primers containing 10 nucleotides (10-mers), primer annealing at 36°C was found to work well for many organisms, including plants (Yu & Pauls, 1994).

A protocol for typing strains of lactic acid bacteria and enterococci which is based on randomly amplified polymorphic DNA (RAPD) fragments, has been developed from a single 10-mer primer. Fingerprints were achieved without the need to isolate genomic DNA. Different conditions of DNA release and amplification were investigated in order to obtain reproducible results and high discrimination among strains. This RAPD protocol was successfully applied for the typing of many strains including *Lactobacillus, Enterococcus* and *Streptococcus* species (Cocconcelli *et al.* 1995). Unfortunately, due to the low stringency of the test, the reproducibility of the RAPD method has been called into question, so although this technique is an extremely powerful tool for such tasks as gene mapping, population and pedigree analysis, phylogenic studies and bacterial strain identification, the reproducibility of these fingerprints can be problematic (Micheli *et al.* 1994).

Using several primers, Zlotkin *et al.* (1997) developed a PCR assay for the detection Gram-positive fish pathogens including *Lactococcus garvieae* , *Streptococcus difficile* and *Vagococcus salmoninarum.* The assay resulted in amplified fragments of 1100bp for *Lactococcus garvieae*, 300bp for *Streptococcus difficile* and 350bp for *Vagococcus salmoninarum.* The specificity and sensitivity of the various primers were evaluated by testing them with the original pathogens and other fish pathogens (*Streptococcus iniae*, *Lactococcus lactis* and *Aeromonas salmonicida*). They were found to be specific for the original pathogens. The assay was found to be sensitive enough to detect bacteria from 1μL of serum or 25mg of tissue. This PCR assay has the potential to, not only diagnose the disease, but to detect these organisms in culture ponds.

Using RAPD and a randomly designed 12-mer primer Miyata *et al.* (1995) demonstrated genomic homogeneity in *Aeromonas salmonicida* subspecies

*salmonicida* and found that it could be used to identify *Aeromonas salmonicida* subspecies *salmonicida* with a high degree of sensitivity and specificity. In a new study, Miyata *et al.* (1996) took a DNA fragment from the RAPD products specific to *Aeromonas salmonicida* subspecies *salmonicida* and cloned it. Then the complete nucleotide sequence of this fragment was determined and a primer set synthesized for the identification of *Aeromonas salmonicida* subspecies *salmonicida* using PCR.

The species-specificity of this primer set was demonstrated by running the RAPD procedure with other fish pathogenic bacteria including *Aeromonas salmonicida* species not subspecies *salmonicida*, *Aeromonas* sp. not *salmonicida*, *Enterococcus seriolicida*, *Edwardsiella tarda*, *Vibrio anguillarum*, *Pasteurella piscicida* and *Yersinia ruckeri*. No PCR products were obtained from any of these species, either alone or as a mixture, and therefore, this technique promises rapid and accurate diagnosis of *Aeromonas salmonicida* subspecies *salmonicida* in fish (Miyata *et al.* 1996).

Factors affecting reproducibility of RAPD fingerprinting were tested using an array of types of DNA polymerases (Schierwater & Ender, 1993) brands of *Taq* DNA polymerase, different thermocyclers, oligonucleotides and bacteria. The results showed that RAPD variations associated with the brand of *Taq* polymerase and the make of the thermocycler may occur, hence an exchange of RAPD data between laboratories should take into consideration the sources of variation (Meunier & Grimont, 1993). Therefore, diagnostic RAPD markers identified in different laboratories may not necessarily be interchangeable and suited for a RAPD data base, unless the same reaction conditions and, in particular, the same type of polymerase, have been used (Schierwater & Ender, 1993; Meunier & Grimont, 1993).

In the RAPD approach to the typing of bacteria, the products that are separated on the agarose gel provide a "fingerprint" of the strain represented by numerous bands in the gel. It is still not clear how effective this technique will be for typing bacteria, because the population genetics of micro-organisms is still poorly understood and sequence variation between species is largely unknown (Priest & Austin, 1993)

### 1.2.7 Pathogenicity studies

Munday *et al.* (1993) studied the pathogenicity of the then *Streptococcus* sp. biovar 1 in two experiments which compared the pathogenicity of *Streptococcus* sp. biovar 1 for rainbow trout versus Atlantic salmon (*Salmo salar*) and rainbow trout versus wild brown trout (*Salmo trutta*). Results indicated that rainbow trout was much more susceptible to streptococcosis than the other fish species.

Kimura & Kusuda (1979) experimentally infected intramuscularly yellowtail with *Streptococcus* species. They sampled at intervals from 10min to 96h, and found that the way in which the organs were affected by the disease was variable. Usually the largest number of bacteria was found first in the kidney, followed by the spleen, liver, blood and intestine. Also, they examined the effect of first injecting the fish with exotoxin of the organism and then challenging them with lowly virulent organisms, highly virulent organisms and exotoxin. It was found that the most long lasting response with the greatest mortality, was in the fish challenged with the bacterial exotoxin, leading them to suggest that exotoxin may play a significant role in the pathogenicity of this disease.

When the pathogenicity of four transformed substrains of *Enterococcus seriolicida* with different agglutination titres was tested in yellowtail by Alim *et al.* (1996).the non-agglutinating sub-strain showed greater virulence (lower $LD_{50}$) than the agglutinating sub-strains. While it was not demonstrated that this property was integrally related to pathogenicity, it appears that repeated subculturing of the organism may bring about a change in the agglutinating property and, concurrently, a decrease in virulence.

The causative agent of streptococcosis in rainbow trout in Italy appears to be closely related to *Enterococcus seriolicida* (now thought to be *Lactococcus garvieae*). It is highly pathogenic for this fish species, with an $LD_{50}$ of $10^2$ cfu/mL (Ghittino *et al.* 1995). A similar organism was also highly virulent for turbot in Spain with $LD_{50}$ of $10^4$ cells (Toranzo *et al.* 1994a). The virulence of two species of streptococci (*S. shiloi* and *S. difficile),* that caused high rates of infection in tilapia and rainbow trout

in Israel, was increased from an $LD_{50}$ of $10^7$ - $10^8$ cfu to $10^2$ - $10^3$ cfu after three passages *in vivo* using brain tissue passage in fish without culture onto agar plates (Eldar *et al.* 1995a).

Beta-haemolytic streptococci causing disease in rainbow trout were found to have a capsule when examined with the india ink method (Yoshida *et al.* 1996; Yoshida *et al.* 1997). Using the phagocytosis assay, chemiluminescence, cell surface hydrophobicity and experimental infection these authors showed that the encapsulated organisms were more virulent than the non-encapsulated organisms of the same isolate. This was deemed to be as a result of resistance to opsonophagocytosis by macrophages (Yoshida *et al.* 1996; Yoshida *et al.* 1997). These streptococci are not similar to the organism now known as *Lactococcus garvieae.*

### 1.2.8 Transmission

Transmission of "streptococcosis" in fish is thought to be horizontal, with infection occurring by direct contact with infected fish or contaminated fish food (Romalde *et al.* 1996). It was found that by introducing diseased golden shiners (*Notemigonus crysleucas*) into an aquarium containing healthy fish, *Streptococcus* sp. was transmitted to the healthy fish (Robinson & Meyer, 1966). Also, infection was established by bathing healthy fish in a suspension of $10^4$ cells/mL (Robinson & Meyer, 1966).

Streptococcal disease affecting tilapia hybrids and rainbow trout in 1984 spread rapidly in cultured fishponds, but horizontal transmission of *Streptococcus* spp. did not occur to common carp (*Cyprinus carpus*) reared in the same community as diseased tilapia, suggesting that this species may not be as susceptible (Eldar *et al.* 1995a).

Jack (1990) undertook a study on the potential of the disease being transmitted vertically via the egg of infected fish over a 14 month period on a farm affected by the disease. Egg monitoring by culturing the surface and the interior of the egg for *Streptococcus* sp. biovar 1 found no evidence of the organism being present.

### 1.2.9 Environmental sources of the causative organisms of streptococcosis

The presumptive environmental source of the organisms (*Streptococcus, Enterococcus seriolicida* or *Lactococcus garvieae*) remains an enigma, but it is thought that they may survive well in the sediment, due to the fact that it is unusual to find infection in trout, especially in under-yearling, held in impervious containers (Munday, 1996).

The *Streptococcus* sp., now believed to be *Lactococcus garvieae,* found in sea water and mud near yellowtail culturing pens in Kitaura Bay, Miyazaki Prefecture was found to be the same as the organism producing the disease in the yellowtail. Contaminated samples of the sea water and mud were collected in winter and summer, which suggested that yellowtail may have abundant opportunities to become infected all year round (Kitao *et al.* 1979). Later, in the same bay, Iwata (1982) found that streptococcal disease in cultured yellowtail may be related to the sardines which were used as feed.

Also, a leech, *Batracobdelloides tricarinata* found in fresh water dams in which rainbow trout were not kept, so, therefore, could not have become infected by rainbow trout, was identified as a potential vector or reservoir for the infection in rainbow trout in South Africa. A *Streptococcus* sp. biochemically and serologically identical to the trout-pathogenic species, was isolated from homogenised samples of the leech. The leech has not yet been recorded as feeding on rainbow trout suggesting that the leech was only a potential reservoir of the rainbow trout pathogenic *Streptococcus* species (now believed to be *Lactococcus garvieae*) in South Africa (Bragg *et al.* 1989b).

### 1.2.10 Humoral response to infection

Immune responses in fish are influenced by many of the factors which affect immunity in homoiothermic vertebrates. These include stress, hormonal changes, seasonal effects, drugs and environmental pollutants (Manning & Mughal, 1985) In addition, as aquatic poikilotherms, fish are particularly susceptible to changes in water quality and to the effects of ambient temperature (Manning & Mughal, 1985).

ELISA was used to compare the humoral immune response of farmed turbot to experimental injection of different strains of bacteria, including the organism (*Enterococcus* sp.) causing the outbreak in the turbot, an *Enterococcus* sp. known to cause disease in trout and an organism identified as *Enterococcus seriolicida* (YT3). As might be expected, the turbot isolate induced the highest levels of antibodies whilst the other two strains, not known to cause disease in turbot, hardly induced antibodies even at the highest dose (Leiro *et al.* 1996).

### 1.3 Treatment

#### 1.3.1 Chemotherapy

Before antimicrobial compounds can be sanctioned for use on fish farms there is a long list of criteria to be fulfilled, including safety, quality control, $LD_{50}$, carcinogenicity, mutagenicity, teratogenicity, stability and efficacy. There is also a suggested code of practice to be observed (Austin, 1985). Also, the use in aquaculture of medically important drugs such as those used to treat tuberculosis, serious staphylococcal infections, typhoid, bubonic plague etc., should be discouraged where possible due to the potential for resistance to develop (Austin, 1985).

##### 1.3.1.1 Dose rates and routes of administration

The chemotherapeutic agents routinely administered to farmed turbot for controlling most bacterial infections are mainly tetracyclines and quinolones (oxolinic acid and flumequine). Erythromycin is only employed in an attempt to control infections by Gram-positive bacteria such as *Streptococcus*-like species (Toranzo *et al*, 1994b). Although the South African strain of *Streptococcus* sp. (now believed to be *Lactococcus garvieae*) isolated from rainbow trout (*Salmo gairdneri*) was sensitive to tetracycline, chloramphenicol and erythromycin, it was found that levels of 75mg oxytetracycline/kg of fish per day added to the feed had little effect on the outcome of the disease (Bragg, 1988).

Katae (1982) applied erythromycin in *in vivo* and *in vitro* trials against streptococcal disease in yellowtail. *In vitro* studies of isolates of *Streptococcus* sp. isolated from diseased fish were tested for the MIC and were found to be susceptible to 0.05 to 0.2u/mL and no resistant strains were detected. *In vivo,* orally administered

erythromycin was easily absorbed with post administration levels peaking at one to three hours. Dosages of 25-50mg/kg/day for 4-7 days were effective against experimental streptococcal infection in yellowtail.

The antibacterial activities of 19 chemotherapeutic agents were tested against 370 strains of a non-haemolytic *Streptococcus* sp. (now believed to be *Lactococcus garvieae*) isolated from cultured yellowtail in Japan in 1986 and 1987 (Aoki *et al.* 1990). Almost 16% of the isolates showed resistance to the macrolide antibiotics lincomycin, tetracycline, and chloramphenicol. The resistance was found to transferable in a small percentage of the isolates. The observed resistance was likely to be attributable to the extensive use of chemotherapeutics in fish farms (Aoki *et al*, 1990).

The macrolides have usually shown strong antibacterial activity against *Streptococcus* species. In yellowtail culture farms in recent years a number of strains of *Streptococcus* sp. have developed resistance to this group of antibiotics (Aoki *et al.* 1990). More recently, the minimum inhibitory concentrations of some newer ionophores (lasalocid, monensin, narasin and salinomycin) were determined *in vitro* for an *Enterococcus*-like (now believed to be *Lactococcus garvieae*) species pathogenic for rainbow trout in Australia. When compared to the macrolide erythromycin, the ionophore narasin was found to be the most inhibitory and monensin the least inhibitory (Carson & Statham, 1993).

### 1.3.1.2 Withdrawal times

Katae *et al.* (1980) studied the distribution, excretion and residue of erythromycin in cultured yellowtails treated for streptococcosis. The levels were distributed in the blood and tissue at different concentrations and the withdrawal times measured as the biological half-life after 10 consecutive doses were as follows:-

Blood 5.00-7.78h
Muscle 7.15-8.17h
Liver 8.37-9.75h
Kidney 14.80-15.63h
Spleen 14.32-15.89h

Levels below the assay limit (0.03-0.08µg/mL) were reached 144-168h post dosing and no evidence of long-term residue was found in any of the tissues tested. Residues of antibiotics in edible portions of fish should be undetectable or below a defined, regulatory level before the fish can be considered fit for human consumption. Tissue levels of these drugs are usually affected by the route of administration, dosage, length of treatment and the water temperature (Jacobsen, 1989). The recommended withdrawal times for salmonids treated with erythromycin, which is the drug of choice for treatment of streptococcosis are 80 days when the water temperature is less than 9°C and 40 days when the water temperature is greater that 9 °C after a dose of 20mg/kg body weight intraperitoneally (Munday, 1994).

To examine the withdrawal times of three antibiotics, oxolinic acid, oxytetracycline and trimethoprim, levels were measured in whole gutted rainbow trout *Salmo gairdneri* Richardson, and in their skin, muscle and blood by high-pressure liquid chromatography at three temperatures (Jacobsen, 1989). The absorption of drugs in rainbow trout was influenced by temperature the experiment was performed at and by the type of drug used (Table 1.10). Jacobsen (1989) recommended that general withdrawal times should be established for all drugs using temperature as an important parameter based on residual quantities in whole gutted trout which usually is the major part that is consumed apart from the bones and head although they may be made into soup stock (Table 1.10).

**Table 1.10 Persistence of oxolinic acid, oxytetracycline and trimetoprim in some rainbow trout tissues.**

| Drug | | Temperature | | |
|---|---|---|---|---|
| | | 6 | 12 | 18 |
| Oxolinic acid | Whole gutted | 22d | ND | 8d |
| | Skin | ND | 8d† | ND |
| | Muscle | ND | 8d† | ND |
| Oxytetracycline | Whole gutted | 47d | ND | 22d |
| | Skin | ND | 52d | ND |
| | Muscle | ND | 22d | ND |
| Trimetoprim | Whole gutted | 36d | ND | 15d |
| | Skin | ND | 57d | ND |
| | Muscle | ND | 8d | ND |

.† no residual drug detected
ND no data given

Jacobsen, (1989) found that to meet the guidelines, the withdrawal time at lower temperatures, as experienced in winter, should be much longer (up to 90 days) for oxytetracycline, which is one of the drugs of choice for the treatment of streptococcosis (Table 1.11).

**Table 1.11 Summary of the recommended withdrawal times in days for drug treatments of fresh water rainbow trout (Jacobsen, 1989)**

| | Oxolinic acid | Oxytetracycline | Trimethoprim |
|---|---|---|---|
| <6 °C | ND | 90d | ND |
| 6 - 12 °C | ND | 70d | ND |
| < 8 °C | ND | ND | 60d |
| > 8 °C | ND | ND | 40d |
| > 12 °C | ND | 60d | ND |
| ≈18 °C | 20d | ND | ND |
| < 18 °C | < 20d | ND | ND |

ND no data

The streptococcal strain isolated from a disease outbreak in flounder, *Paralichthys olivaceous*, in Korea was resistant to ampicillin, erythromycin, gentamicin, tetracycline and vancomycin (Kim & Lee, 1994). This information would suggest that this organism maybe, in fact, different to the organism now believed to be *Lactococcus garvieae*, because *Lactococcus garvieae* is usually sensitive to ampicillin, erythromycin and vancomycin.

## 1.4 Prevention

### 1.41 Immunotherapy

#### 1.4.1.1 Active immunisation

Field experience has shown that therapeutic measures are commonly ineffective when fish are infected by Gram-positive cocci (Ghittino *et al*, 1996). Therefore, the development of vaccines is essential as it offers the best possibility for control of this type of disease (Ghittino *et al*, 1996).

Many factors have an impact on the effectiveness of a fish vaccine, in particular, the route of delivery can substantially affect the degree of protection conferred *in vivo* (Toranzo *et al*, 1995b). This may, in fact, reflect the efficiency of transferring the

immunogenic constituents of the vaccine to the recognition and effector components of the fish immune system. Toranzo *et al.* (1995b) evaluated both bath immersion and intraperitoneal injection of an *Enterococcus* sp., that was phenotypically similar to both *Enterococcus seriolicida* and *Lactococcus garvieae* (Toranzo *et al.* 1994a; Toranzo *et al.* 1994b), in cultured turbot. They found that only the injection route affected the level of in terms of protection and of phagocytic activity (Toranzo *et al*, 1995b). In this work by Toranzo *et al.*, a toxoid-enriched whole cell bacterin of the *Enterococcus* sp. was used in cultured turbot to measure the effectiveness against enterococcal infection. Also included in the study was the addition of the β-glucan immunostimulant Trouvitol plus® to the diet of half of the fish to examine the efficacy of the addition of immunostimulants during vaccine trials (Toranzo *et al.* 1995b). The results showed that the addition of the immunostimulant had no effect on the outcome of the vaccine trial (Toranzo *et al.* 1995b).

Iida *et al*, (1982) tested the efficacy of oral vaccination for the control of streptococcosis disease in cultured yellowtail and found the vaccination effect to be very short, giving only 2 weeks protection.

Sakai *et al.* (1987) found that rainbow trout vaccinated against streptococcosis by immersion or intraperitoneal methods showed less protective immunity using the immersion method. The relative percent survival (RPS) with the injected vaccine was 90% while the RPS for immersion was 70%. The advantage of the immersion method is that it is faster and easier to use for fish farmers. Later, Sakai *et al*, (1991) measured the chemiluminescent response of leucocytes from the anterior kidney to compare routes of administration of streptococcal vaccine in rainbow trout. The peak chemiluminescent response after immersion was 98mV, whilst after injection it was 179mV 25 days after vaccination, showing that the bactericidal activity of the phagocytes was higher using the injection route

Akhlaghi *et al*, (1996) compared active immunisation of rainbow trout by either immersion or intraperitoneal injection with formalin-killed streptococcal cells prepared from *Streptococcus* sp. biovar 1 cells using the original Tasmanian isolate which is now believed to be *Lactococcus garvieae*. The fish were monitored over a three

month period and the results showed that the intraperitoneal route of immunisation gave the best protection after the trout were challenged with the virulent streptococci from which the vaccine was prepared (Table 1.12)

**Table 1.12 Relative percent survival after active immunisation with streptococcal antigen by two methods (Akhlaghi *et al.* 1996)**

| Technique | Immersion | Intraperitoneal injection |
|---|---|---|
| One month | 11% | 88% |
| Two months | 5% | 38% |
| Three months | 0 | 36% |

Ghittino *et al*, (1995) vaccinated rainbow trout with a formalin-inactivated whole cell streptococcal vaccine prepared from an organism phenotypically similar to *Enterococcus seriolicida* and, therefore, probably *Lactococcus garvieae.* The fish, which were vaccinated via the intraperitoneal route, showed that the immunity, established at the third week post-vaccination, was still complete more than 4 months later, since no mortalities were recorded then after challenging the fish with bacterial concentrations of 100 times the original $LD_{50}$ via the intraperitoneal route (Ghittino *et al*, 1995). These results suggest that active immunisation via the intraperitoneal route is potentially useful in protecting fish from streptococcosis for at least the time at which it is most prevalent, during periods of higher water temperature in summer and autumn.

Bercovier *et al.* (1997b) used formalin-killed bacteria as vaccines against *Streptococcus iniae* and *Lactococcus garvieae* infections in farmed rainbow trout and found that a single intraperitoneal injection resulted in protection for 3 to 4 months. Under field conditions, the mortality of non-vaccinated trout was greater than 50% whilst mortality in the vaccinated fish was less than 5%. Also, these vaccines, when adjuvanted with oil emulsions, protected the fish for up to 6 months.

### 1.4.1.2 Passive immunisation

Akhlaghi *et al*, (1996) found that, despite a variable level of early protection against streptococcosis (caused by the organism now believed to be *Lactococcus garvieae*), in rainbow trout after passive immunisation with sheep, rabbit and fish anti-

streptococcal antibodies (ASA), sheep afforded the best protection with low levels of protection with rabbit and fish ASA [see 1.4.1.1] and passive and active modes of immunisation gave similar levels of protection.

**Table 1.13 Relative percent survival after passive immunisation with three types of anti-streptococcal antisera (Akhlaghi *et al.* 1996)**

| | Sheep ASA | Rabbit ASA | Fish ASA |
|---|---|---|---|
| One month | 88% | 50% | 0 |
| Two months | 33% | 7% | 7% |
| Three months | 13% | 0 | 6% |

Akhlaghi *et al*, (1993) experimented with two different intraperitoneal sites for the injection of vaccine into fish to compare the effectiveness using an anti-*Vibrio* antisera and found that there was no real difference between intraperitoneal injection being performed immediately anterior to the vent or anterior to the pelvic fin.

### 1.4.1.3 Modulators of immune response

Immunomodulators have become important in fish culture for heightening the activity of non-specific defence mechanisms and thus conferring protection against disease, particularly in young fish (Anderson, 1992; Jeney & Anderson, 1993). Immunomodulators can be administered on their own, or in conjunction with vaccination (before, with, or after) to enhance the specific immune response to the vaccine, raising the levels of circulating antibodies and the numbers of plaque-forming cells (Anderson, 1992). In one observation (Jeney & Anderson, 1993) in vitro tissue culture was used to test the immunomodulatory capacity of a quaternary ammonium compound, a polypeptide ISK and levamisole. This was found to be an efficient method of pre-screening substances before *in vivo* experiments. Recently, the oral administration to rainbow trout of the fermented product of chicken eggs (EF203) has been shown to produce protective immunity against the Gram-positive organism *Renibacterium salmoninarum*, via enhanced phagocytic activity (Sakai *et al*, 1995).

Resistance to *Enterococcus seriolicida* infection in yellowtail was enhanced by the administration of a peptidoglycan derived from *Bifidobacterium thermophilum*. The peptidoglycan was administered at a rate of 0.2mg $kg^{-1}$ fish body weight, which caused

an increase in the phagocytic activity of the pronephros cells, one of the important factors in non-specific defence mechanisms (Itami *et al*, 1996). The survival rate of the P-G fed fish was 86% compared with 43% in the control group (Itami *et al*, 1996).

The polysaccharides schizophyllan and scleroglucan derived from *Schizophyllum commune* and *Sclerotium glucaniicum* respectively, which are known as anti-tumour substances and as stimulants of the non-specific immune system in mammals (Robertsen *et al*, 1994; Raa, 1996), were also used to demonstrate a protection against streptococcal (the organism now believed to be caused by *Lactococcus garvieae*) infection by stimulating phagocytic activity of pronephros cells of yellowtail (Matsuyama *et al*, 1992). As well, enrichment of the standard semi-moist fish diet with the commercial preparation Trouvitol plus®,which contains ß-glucan from yeast, induced a non-specific defence mechanism in cultured turbot. However, efficacy, in conjunction with vaccine against challenge with *Enterococcus* sp. was not demonstrated (Toranzo *et al*, 1995b).

Ninomiya *et al.* (1995) found that chemotactic activity of yellowtail leucocytes was enhanced by oral administration of quillaja saponin, an extract of the soap tree, *Quillaja saponaria*. The migratory response was significantly increased with dosages of 5 and 50mg $kg^{-1}$ body weight and, when measured over a period of 24 to 384h, was most increased at 48 and 96h.

### 1.4.2 Fish management

#### 1.4.2.1 Farming techniques

Disease is particularly prevalent in intensive aquaculture where fish are stocked at high intensities, and subjected to stress, and where basic hygiene necessary for maintaining good water quality may be overlooked (Austin & Allen-Austin, 1985). Prevention of streptococcosis is better and cheaper than the cure and measures such as isolation of any suspected fish and a clean uncontaminated water supply are recommended (Roode, 1977).

Contaminated sediment in the farmed fish environment may contribute greatly to the risk of infection, therefore, the use of concrete ponds and raceways that do not provide a refuge for the causative organism, may help to control the disease streptococcosis (Carson & Munday, 1990).

Raised water temperature appears to be conducive to the proliferation of cases of streptococcosis, but is difficult to control in most situations, therefore it would be necessary to modulate stocking densities in line with temperature rises and also to create an impervious barrier between potential bacteria-harbouring sediment and the fish (Munday *et al.* 1993; Munday, 1996). Lowering the stocking densities in sea cages and situating sea cages containing infected fish at least 100 metres from cages holding uninfected fish are other methods that can be implemented (Munday, 1996). Hygienic measures such as footbaths should also limit the spread of disease (Munday, 1985)

### 1.4.2.2 Disinfection

*Streptococcus* sp. biovar 1 was found to be extremely resistant to iodophors and was unaffected at 300ppm for a 30 minute contact time, but disinfection with benzalkonium chloride at 1000ppm for 20 minutes, sodium hypochlorite at 500ppm for 2 minutes or chloramine T at 750ppm for 20 minutes was effective (Carson & Munday, 1990).

There was one instance when 0.5% formalin overnight failed to completely inactivate the bacterium and disease occurred in inoculated fish (D. Jack pers. comm.).

**Table 1.14** Active concentrations of disinfectants against *Streptococcus* sp. biovar 1 with 2 minute contact time (B. Pike pers. comm.).

| Chemical | Concentration | Activity |
|---|---|---|
| Hypochlorite | 600ppm | free chlorine |
| Benzalkonium chloride | 500ppm | |
| Malachite green | relatively ineffective | |
| Iodophors | relatively ineffective | |

Ozone disinfection was applied to kill *Enterococcus seriolicida* in sea water to determine the relationship between total residual oxidant (TRO) doses and the contact times needed to inactivate the bacterial fish pathogen. The 99% inactivation point was achieved at a concentration of 0.111mg/L and the mean 99 and 99.9% killing concentration-contact time (C . *t*) products were 0.123 and 0.186mg · min/L (Sugita *et al.* 1992).

### 1.5 Commercial loss

#### 1.5.1 Direct loss to the industry

In 1974, in Japan, an epizootic caused by *Lactococcus garvieae* (but at the time studies placed the bacterium in the genus *Enterococcus*) produced heavy losses among cultured yellowtail (Kusuda & Salati, 1993). About 25% of total production of mariculture in Japan has been lost to fish diseases in recent years and *Lactococcus garvieae* (formerly reported as *Enterococcus seriolicida* or *Streptococcus* sp.) infection of yellowtail causes the greatest financial loss, because it affects fish of marketable size (Kusuda, 1992).

In 1989 the reported commercial loss in yellowtail was approximately 8,240 ton, 10 ton for sea bream (*Pagrus major*), 88.3 ton for saurel (*Trochurus japonicus*) and 88.3 ton for flounder (*Paralichthys olivoceus*) (Kusuda & Salati, 1993).

Serious economic losses, attributed to streptococcosis have been reported in yellowtail, Japanese eels and rainbow trout (Bragg *et al.* 1989a). In South Africa and Australia rainbow trout is the commercial species most affected, (Carson & Munday, 1990) while in Japan major losses are reported in farms growing ayu and yellowtail (Kusuda & Salati, 1993)..

Serious economic loss of cultured turbot in northern Spain due to streptococcal (now believed to be lactococcal) disease was not so much the mortality (0.12 - 4%), but the wide size range of fish affected, low growth rate due to infection and the unpleasant external appearance making the fish unmarketable (Toranzo *et al.* 1994a).

### 1.5.2 Indirect loss to the industry

The indirect loss to the industry has resulted from decreased choice of species (especially rainbow trout) which cannot be cultured in warmer waters in endemic areas (Jack, 1990; Munday *et al.* 1993).

## *CHAPTER TWO*: GENERAL INTRODUCTION

In recent years the intensive culture of fish for the commercial market has increased greatly and has become an important part of the Australian primary industry. Encouraged by the Tasmanian State Government through its agencies, the Department of Sea Fisheries and the Tasmanian Development Authority, several million dollars have been invested in mariculture in Tasmania (Garland & Carson, 1987). Rainbow trout (*Oncorhynchus mykiss*), previously termed *Salmo gairdneri*, have been grown in freshwater facilities in Tasmania since 1898. They were initially produced to stock lakes and streams for recreational fishermen (Clements, 1988). Since the early 1960s, the species has been intensively farmed for the culinary market, the first farm operating at Bridport (Fig 3.1) (A. Purves, 1997 pers. comm.). Production figures for trout and other salmonids are sketchy as information from the growers has been difficult to obtain. The available data on trout given in Figure 2.1

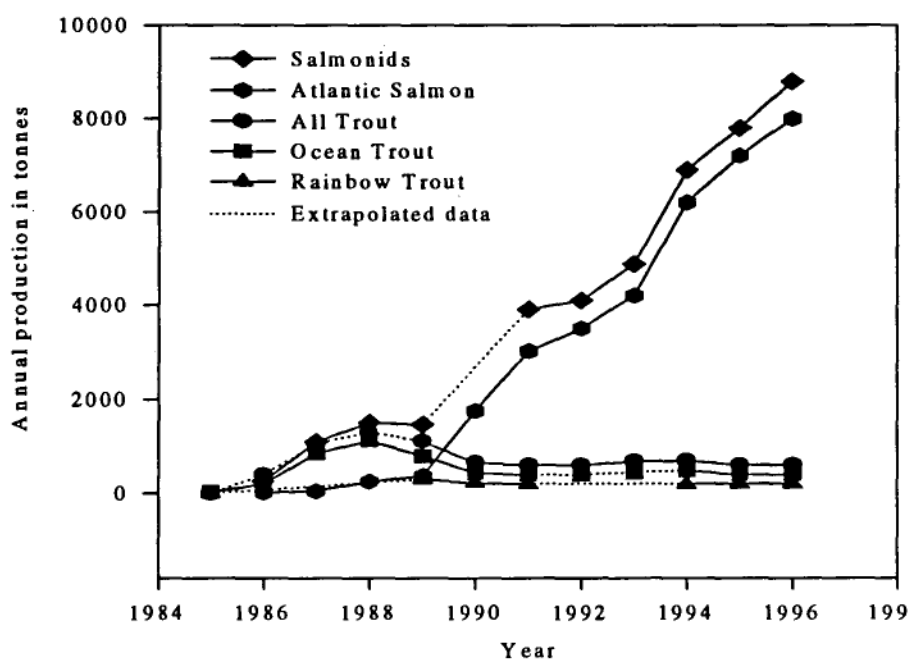



**Figure 2.1** Production figures for salmonids in Tasmania (Treadwell *et al.* 1991; D. O'Sullivan, 1997, pers. comm.; Tasmanian Salmonid Growers Association, 1997, pers. comm.)

were supplied by O'Sullivan & Kiley (1996) and D. O'Sullivan (1997, pers.comm.). The Atlantic salmon production figures (Figure 2.1) were supplied by the Tasmanian Salmonid Growers Association (1997, pers. comm.).

Initially, farming of trout was undertaken in fresh water only, but from 1969 small scale attempts were made to farm this species in sea water in the Tamar river in northern Tasmania (A. Purves, 1997 pers. comm.), with commercial production commencing initially in southern Tasmania in 1985. Production is now concentrated in Macquarie Harbour, a low salinity embayment on the west coast of Tasmania (Purser, 1992).

In the late 1970's, a disease, characterised by either peritonitis or septicaemia, occurred in a freshwater rainbow trout farm at Bridport, Tasmania (Figure 4.1.1). The organisms which were isolated were identified at the time as two similar strains of *Streptococcus* spp., a genus which was already known to cause the disease streptococcosis in fish in other countries, especially Japan and South Africa (Munday & Copland, 1981).

There is not much published documentation of the history of streptococcosis in Tasmania. After the Bridport incident, there were sporadic outbreaks of streptococcosis at other freshwater trout holding facilities belonging to the same company. These were related to spawning and are best described as spawning-associated peritonitis due to *Vagococcus* sp. (B. Munday, 1997 pers. comm.). Then a large outbreak of streptococcosis, in which fish mortalities reached 75% in some ponds, occurred at the Cressy facility (Figure 4.1.1) of the same company in the summer of 1985/86. The outbreak was thought to be related to a combination of events including high rainfall resulting in flooding, contamination of water with agricultural run-off containing faecal material, lack of fresh water resulting in oxygen starvation and raised water temperature. This combination of factors resulted from the need to close the water intake and recirculate water during a summer flood (S. Pitney, 1986 pers. comm.).

Not long after commercial sea farming commenced in Tasmania in 1985, outbreaks of streptococcosis occurred at a number of farms in southern Tasmania, especially during stress periods of acclimatisation to increased salinity. The origin of these fish was usually from the areas were the disease was endemic, for example, the Cressy and Bridport facilities already mentioned (B. Munday, 1997 pers. comm.; J. Purser, 1997 pers. comm.). All these outbreaks were due to a Gram-positive coccus which was given the identifier *Streptococcus* sp. biovar 1 (Carson & Munday, 1990).

In Tasmania, the major host for the disease streptococcosis is rainbow trout (*Oncorhynchus mykiss*) previously known as *Salmo gairdneri* (Kendall, 1988; Smith & Stearley, 1989).

Prior to the first outbreak of streptococcosis in Tasmania, existing conditions could have been conducive for the disease to occur. In the absence of any earlier disease, it must be presumed that the organism was somehow introduced into the local trout population from elsewhere. However, there remains a slight possibility that it had existed undetected until environmental conditions were suitable for an outbreak of overt disease. As there had been no new rainbow trout stock movement to Tasmania from elsewhere since 1982 (W. Fulton, 1997 pers. comm.), it is unlikely that the source was trout farms interstate or overseas.

Potential sources of the causative organism are as follow:-

1) The feed.
2) Faecal contamination of waterways by domestic animals (cattle, sheep, pigs & chickens).
3) Faecal contamination of waterways by humans.
4) Faecal contamination of waterways by wild/feral terrestrial animals
5) Contamination by wild fish in the water supply.

As mentioned previously, the Gram-positive organism causing severe loss in the rainbow trout industry in Tasmania was given the epithet *Streptococcus* biovar 1 (Carson & Munday, 1990). In 1993 the organism was renamed as an *Enterococcus*-like organism (Carson *et al.* 1993) after Kusuda *et al.* (1991) had identified a similar

organism causing disease in yellowtail (*Seriola quinqueradiata*) in Japan and named it *Enterococcus seriolicida* (ATCC 49156). It is notable that at that time, Carson *et al.* (1993) did not suggest that the Australian *Enterococcus*-like organism was actually *Enterococcus seriolicida.*

The essence of our research project reported here was to locate a possible source of the pathogen in the aquatic environment of the fish farms and also to investigate the potential contamination of the environment from neighbouring agricultural enterprises. As streptococci, enterococci and, indeed, lactococci, are part of the normal flora of numerous members of the animal kingdom, it is possible that a non-aquatic animal could have been the original source of this fish pathogen.

Streptococcosis, as a syndrome, typically produces a disease in which the fish display signs of septicaemia, including darkening of the skin, bi-lateral exophthalmia, anorexia and lethargic, erratic, spiral-swimming. Ultimately, death usually occurs within hours or days, although chronic infection is not unknown.

The outline of some the research undertaken in this study is summarised below:-

- Chapter three covers the development of suitable selective media for the isolation of organisms similar to those producing streptococcosis in Tasmania and Japan.

- Chapter four, part one, explores the methods used to sample the environment at the sites of the original outbreaks. It also documents the characteristics of the 61 isolates collected from the environmental sites along with ten Australian isolates of *Streptococcus* sp. biovar 1 with *Enterococcus seriolicida* (ATCC 49156), *Enterococcus seriolicida* YT3, *Lactococcus garvieae* (LMG 8893) and *Lactococcus garvieae* (MPL 94-4127.2).

- Chapter four part two describes the use of the molecular biology technique of random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) to differentiate between some of the environmental species and the type strains, and to compare the patterns from the ten Australian reference isolates of *Streptococcus*

sp. biovar 1 and one each of *Enterococcus seriolicida* (ATCC 49156), *Lactococcus garvieae* (LMG 8893).

- Chapter five contains the results of "in vivo" studies on the pathogenicity of one of the Tasmanian isolates of *Streptococcus* sp. biovar 1 and the isolate of *Lactococcus garvieae* (MPL 94-4127.2) isolated from a case of bovine mastitis in Tasmania.

- The final chapter discusses the results obtained from these projects and the outcome of the original hypothesis that the Australian isolate of *Streptococcus sp.* biovar 1 was already present in the environment in Tasmania.

## *CHAPTER THREE*: DEVELOPMENT OF MEDIA FOR THE ISOLATION OF ENVIRONMENTAL GRAM-POSITIVE COCCI

### Introduction

At the time of commencing this study (1993), it was hypothesised that the causative organism of streptococcosis in rainbow trout in Tasmania was already present in the environment, and may even have been part of the normal flora of the local native or introduced fauna. The organism causing disease in rainbow trout in Tasmania was, at the time, thought to be a *Streptococcus* species and had been given the temporary epithet *Streptococcus* sp. biovar 1 (Carson & Munday, 1990). Subsequently, it was suggested that the causative agent of streptococcosis in Tasmania and South Africa was an *Enterococcus* species (Carson *et al.* 1993). Although this work had been predated by that of Kusuda (1991), who gave the name *Enterococcus seriolicida* to the organism causing streptococcosis of yellowtail in Japan, the above authors did not directly equate *Streptococcus* sp. biovar 1 with *Enterococcus seriolicida.*

Prior to sampling the environment, the persistence of the organism in sediment, water and faeces, was examined over an extended period. The organism (*Streptococcus* sp. biovar 1) was introduced in small numbers to samples of sterile sediment, water and faeces and then re-isolated for at least 4 weeks afterwards.

Naturally, samples from the environment contain many organisms, most of which will grow on non-selective media such as horse blood agar (HBA), especially coliforms, so it was necessary to develop selective media that would enrich the streptococci and enterococci and inhibit other organisms. A medium had already been developed in South Africa for the selective isolation of streptococci from contaminated samples, and had been used in a surveillance programme for the presence of the *Streptococcus* in the leech *Batracobdelloides tricarinata* (Bragg *et al.* 1989a; 1989b).

So the next step was to try and find a selective medium that supported the growth of *Streptococcus* sp. biovar 1., and inhibited other organisms likely to be present in the environmental samples, including multi-resistant bacteria, such as *Pseudomonas* and *Staphylococcus aureus* (MRSA) and yeasts and fungi. Ten isolates of the

*Streptococcus* sp. biovar 1 were obtained from Dr Jeremy Carson of the Department of Primary Industry, Mount Pleasant Laboratories. These isolates are summarised in Table 3.1.

**Table 3.1 Isolates of Australian *Streptococcus* sp. biovar 1 used in this study**

| Culture number | Accession number | Serotype |
|---|---|---|
| 6 | V87/1309-1 | 01 |
| 9 | 88/3910-15 | 02 |
| 16 | 86/0290 | 01 |
| 17 | 89/1592-1 | 01 |
| 20 | 88/0756 | 01 |
| 21 | 88/3910-5 | 01 |
| 28 | 88/3625-1 | 01 |
| 36 | V83/35051-1 | 01 |
| 38 | 88/3425-6 | 02 |
| 39 | 88/3910-9 | 02 |

Prefix "V" denotes isolate from Victoria.

## Materials and Methods

### 3.1 Selective and enrichment media

The use of the medium developed by Bragg *et al.* (1989a) was based on the concept that the causative organism was a streptococcus. This theory was later refuted with the suggestion being that the organism was instead an enterococcus-like bacterium (Carson *et al.* 1993). Therefore, the study included a broth medium using one of Bragg's recipes, a modification of Bragg's agar plate medium and two recipes, a modified Oxoid Streptococcus selective media (0.01% triphenyl tetrazolium chloride [TTC] added), and a broth medium containing Streptococcus Selective supplement (Oxoid SR126). Also, because it was later suggested that the organism was, in fact, an enterococcus, the study included two recipes, derived from the method of Slanetz and Bartley (Slanetz & Bartley, 1957), for the selective isolation of enterococci from water, sewage and faeces. The formulae and methods of preparation for the three solid media and three enrichment broths are documented in the Appendix A1 to A6.

For ease of reporting, the media were designated by an abbreviation of their titles, hence Bragg's streptococcal selective agar and selective broth were called BSSA and

BSSB respectively. Modified streptococcal selective agar and broth titled MSSA and MSSB respectively, and Slanetz and Bartley enterococcal selective agar and broth titled SBESA and SBESB respectively. A non-selective medium, (HBA), was also used in the experiment.

### 3.1.1 Growth trials

Initially, an experiment was conducted to test for the ability of the target organism to survive in environmental samples. Samples of non-chlorinated water, faeces and mud were sterilised by autoclaving in culture tubes in 2.5mL aliquots. Cultures of the Australian isolates of *Streptococcus* sp. biovar 1 (Table 3.1) were inoculated into theses samples and the samples incubated at room temperature and sub-cultured regularly onto HBA over a period of at least 4 weeks.

To test the ability of each medium to support the growth of the target organism, all of the media (solid and broth) were inoculated with two strains of *E. faecalis* and one *E. faecium* and one of *Streptococcus bovis* as well as the ten Australian isolates in Table 3.1 of what was then known as *Streptococcus* sp. biovar 1 at a concentration of 0.1mL of approximately $10^4$ to $10^5$ cfu/mL and incubated aerobically overnight at 35°C. The plates were examined for typical colonies and reincubated for a further 24h. Each of the broths were subcultured onto each plates BSSA, MSSA, SBESA, HBA and incubated aerobically overnight at 35°C.

### 3.1.2 Selectivity trials

Organisms used in the selectivity trial included *Aeromonas hydrophila, Pseudomonas aeruginosa,* Methicillin-resistant *Staphylococcus aureus,* a yeast and a filamentous fungus that were not identified. Although most of these organisms were not typical fish pathogens, they may possibly exist in the environment, and were chosen for the nature of their resistance to most antimicrobial substances, the purpose of this experiment being to select a medium that will support the growth of the target species, but eliminate most contaminants. Loopfuls of each of these cultures were streaked onto the solid agar and emulsified into the broth and incubated aerobically overnight at 35°C. The plates were examined for the presence of any growth and reincubated for a further 24h. Each of the broths were subcultured onto plates BSSA,

MSSA, SBESA and HBA and the plates incubated aerobically overnight at 35°C and examined for growth.

Originally, the organisms were held indefinitely at -70°C in a suitable broth such as brain heart infusion containing 15% glycerol. The broths were inoculated with the organism to be maintained, and then transferred to a labelled cryotube in the -70°C freezer. Ultimately they were transferred to a commercial system called Protect ™ (Technical Services Consultants) especially designed for this type of storage.

## Results

### 3.1 Selective media

#### 3.1.1 Growth trials

The samples of water, faeces and mud were all positive for growth of the target organism after more than 4 weeks incubation at room temperature. The results of the evaluation of three types of selective agar using the ten isolates of *Streptococcus* sp. biovar 1, three *Enterococcus* spp. and one Group D streptococcus (*Streptococcus bovis*) are summarised in Table 3.2. Growth was evaluated semi-quantitatively as no growth (NG), light growth (+),moderate (++), heavy (+++) through to very heavy growth (++++).

**Table 3.2 Primary growth on selective agar**

| | BSSA | MSSA | SBESA |
|---|---|---|---|
| MP6 | ++++ | ++++ | ++ |
| MP9 | ++++ | ++++ | ++ |
| MP16 | ++++ | ++++ | +++ |
| MP17 | ++++ | ++++ | +++ |
| MP20 | ++++ | ++++ | ++ |
| MP21 | ++++ | ++++ | ++ |
| MP28 | ++++ | ++++ | ++ |
| MP36 | ++++ | ++++ | ++ |
| MP38 | ++++ | ++++ | ++ |
| MP39 | ++++ | ++++ | ++ |
| *E. faecalis* | ++++ | ++++ | ++++ |
| *E. faecalis* | ++++ | ++++ | ++++ |
| *E. faecium* | ++++ | ++++ | +++ |
| *S. bovis* | +++ | +++ | +++ |

**Table 3.3 Growth after subculture from BSSB**

| | BSSA | MSSA | SBESA | HBA |
|---|---|---|---|---|
| MP6 | ++++ | ++++ | ++ | ++++ |
| MP9 | ++++ | ++++ | ++ | ++++ |
| MP16 | ++++ | ++++ | ++ | ++++ |
| MP17 | ++++ | ++++ | ++ | ++++ |
| MP20 | ++++ | ++++ | ++ | ++++ |
| MP21 | ++++ | ++++ | ++ | ++++ |
| MP28 | ++++ | ++++ | ++ | ++++ |
| MP36 | ++++ | ++++ | ++ | ++++ |
| MP38 | ++++ | ++++ | ++ | ++++ |
| MP39 | ++++ | ++++ | ++ | ++++ |
| *E. faecalis* | ++++ | ++++ | +++ | ++++ |
| *E. faecalis* | ++++ | ++++ | +++ | ++++ |
| *E. faecium* | ++++ | ++++ | +++ | ++++ |
| *S. bovis* | +++ | ++++ | ++ | ++++ |

Table 3.2 demonstrates that the agars BSSA and MSSA both showed good growth results for the target organisms and other similar species, whilst the enterococcus selective (SBESA) medium showed slightly poorer growth with the target organisms, although the selected *Enterococcus* spp. grew well, as would be expected since it is designed for the isolation of these organisms.

**Table 3.4 Growth after subculture from MSSB**

| | BSSA | MSSA | SBESA | HBA |
|---|---|---|---|---|
| MP6 | ++++ | ++++ | ++ | ++++ |
| MP9 | ++++ | ++++ | ++ | ++++ |
| MP16 | ++++ | ++++ | ++ | ++++ |
| MP17 | ++++ | ++++ | ++ | ++++ |
| MP20 | ++++ | ++++ | ++ | ++++ |
| MP21 | ++++ | ++++ | ++ | ++++ |
| MP28 | ++++ | ++++ | ++ | ++++ |
| MP36 | ++++ | ++++ | ++ | ++++ |
| MP38 | ++++ | ++++ | ++ | ++++ |
| MP39 | ++++ | ++++ | ++ | ++++ |
| *E. faecalis* | ++++ | ++++ | +++ | ++++ |
| *E. faecalis* | ++++ | ++++ | +++ | ++++ |
| *E. faecium* | ++++ | ++++ | +++ | ++++ |
| *S. bovis* | +++ | ++++ | ++ | ++++ |

After subculture of the three selective enrichment broths onto the three selective agars as shown in Tables 3.3 to 3.5, it can be observed that the agars BSSA and MSSA demonstrated similar recovery rates of the target organism and, although the SBESA

performed well with the *Enterococcus* spp., it was less effective with the target organism.

**Table 3.5 Growth after subculture from SBESB**

| | BSSA | MSSA | SBESA | HBA |
|---|---|---|---|---|
| MP6 | ++++ | ++++ | ++ | ++++ |
| MP9 | ++++ | ++++ | ++ | ++++ |
| MP16 | ++++ | ++++ | ++ | ++++ |
| MP17 | ++++ | ++++ | ++ | ++++ |
| MP20 | ++++ | ++++ | ++ | ++++ |
| MP21 | ++++ | ++++ | ++ | ++++ |
| MP28 | ++++ | ++++ | ++ | ++++ |
| MP36 | ++++ | ++++ | ++ | ++++ |
| MP38 | ++++ | ++++ | ++ | ++++ |
| MP39 | ++++ | ++++ | ++ | ++++ |
| *E. faecalis* | ++++ | ++++ | +++ | ++++ |
| *E. faecalis* | ++++ | ++++ | +++ | ++++ |
| *E. faecium* | ++++ | ++++ | +++ | ++++ |
| *S. bovis* | +++ | ++++ | ++ | ++++ |

### 3.1.2 Selectivity trials

The results of the evaluation of six types of media using the organisms *Aeromonas hydrophila, Pseudomonas aeruginosa,* methicillin resistant *Staphylococcus aureus,* and an unidentified yeast and fungus are summarised in Tables 3.6 to 3.9. Growth was semi-quantified as before as no growth (NG), light growth (+),moderate (++), heavy (+++) through to very heavy growth (++++).

**Table 3.6 Primary growth on selective and non-selective agar plates**

| | BSSA | MSSA | SBESA | HBA |
|---|---|---|---|---|
| *P. aeruginosa* | ++ | ++ | ++ | ++++ |
| *S. aureus* | ++ | + | NG | ++++ |
| *A. hydrophila* | + | + | ++ | ++++ |
| Yeast | +† | +† | NG | ++++ |
| Fungus | +† | NG | NG | ++++ |

† after prolonged incubation

These results showed that there was no great difference in the selectivity of the broths except that the Slanetz and Bartley broth (SBESB) was highly selective against all the potential contaminants, but as *Streptococcus* sp. biovar 1 did not grow well on solid media after incubation in SBESB, BSSB was selected as the broth medium. The

**Table 3.7 Growth after subculture from BSSB**

| | BSSA | MSSA | SBESA | HBA |
|---|---|---|---|---|
| *P. aeruginosa* | NG | NG | NG | + |
| *S. aureus* | + | NG | NG | + |
| *A. hydrophila* | NG | NG | NG | NG |
| Yeast | NG | NG | NG | NG |
| Fungus | NG | NG | NG | NG |

**Table 3.8 Growth after subculture from MSSB**

| | BSSA | MSSA | SBESA | HBA |
|---|---|---|---|---|
| *P. aeruginosa* | + | NG | NG | ++++ |
| *S. aureus* | NG | NG | NG | NG |
| *A. hydrophila* | NG | NG | NG | NG |
| Yeast | NG | NG | NG | NG |
| Fungus | NG | NG | NG | NG |

**Table 3.9 Growth after subculture from SBESB**

| | BSSA | MSSA | SBESA | HBA |
|---|---|---|---|---|
| *P. aeruginosa* | NG | NG | NG | NG |
| *S. aureus* | NG | NG | NG | NG |
| *A. hydrophila* | NG | NG | NG | NG |
| Yeast | NG | NG | NG | NG |
| Fungus | NG | NG | NG | NG |

selective agar plates were also similar in inhibiting the potential pathogens, although MSSA performed marginally better and so was chosen as the selective agar for the study. In summary: it was found that using a primary enrichment broth medium containing 100µg/mL of nalidixic acid and 200µg/mL of sodium azide followed by subculture onto agar containing 10µg/mL colistin sulphate, 5µg/mL oxolinic acid and 0.01% TTC was the most satisfactory for the enhancement of the *Streptococcus* sp. biovar 1 or *Enterococcus* sp., and for the inhibition of potential environmental contaminants.

**Discussion**

The need to prepare good media to selectively support the growth of the Australian isolates was of paramount importance due to the type of samples being cultured. Although Bragg *et al.* (1989a) had developed a selective media for the field isolation of the strain of streptococcus causing disease in South Africa, it was not known at the

time if their organism was the same as the Australian strain. In the early stages of this work, the organism was suspected to be enterococcus-like (Carson *et al.* 1991) and for this reason the *Enterococcus* selective media (broth and solid) of Slanetz and Bartley (1957) were also included in the trial, although these media were designed mainly for the isolation of *Enterococcus faecalis*. *E. faecalis* grew well on the solid medium which, however, tended to inhibit the growth of the *Streptococcus* sp. biovar 1, probably due to the presence of azide.

Kitao *et al.* (1979) used enrichment in a broth containing sodium azide and then subculture onto an *Enterococcus faecalis* medium when isolating *Streptococcus* from the seawater near yellowtail culturing pens. It is not known if this organism was similar to the Australian isolates, although it is more likely to be more closely related to *E. faecalis* and *E. faecium* as suggested by Kusuda *et al.* 1976 and Minami *et al.* (1979), and hence the ability to grow on *Enterococcus faecalis* medium.

The modified streptococcal selective media (MSSM) was included in the experiment as it contained readily-available supplements to inhibit the growth of most non-streptococcal organisms. The recipe was modified by the addition of the TTC to detect the intended enterococcal-like organisms because the TTC is converted to fomazone producing a red or magenta colour in the colony.

This part of the work has resulted in the successful production of two media that were able to selectively isolate and enrich *Streptococcus* sp. biovar 1 and at least three enterococci and one other streptococcus from heavily-contaminated sources.

## *CHAPTER FOUR*: COMPARATIVE "IN VITRO" STUDIES OF SELECTED ISOLATES

### 4.1 Isolation and phenotypic characterisation of selected isolates

**Introduction**

*Enterococcus seriolicida* (YT3) was proposed by Kusuda (1991) as a novel species of *Enterococcus*. His finding was based on the biochemical characteristics, determination of the moles percent of guanine and cytosine (the G+C ratio) and DNA-DNA hybridisation studies. A type culture provided to the American Type Culture Collection was given the number ATCC 49156. Late in 1994, after an Australian Quarantine and Inspection Services (AQIS) permit to import the culture was obtained, Professor Riichi Kusuda from the fish disease laboratory at Kochi University in Japan supplied a freeze dried culture of his *Enterococcus seriolicida* YT3. Preliminary studies on the isolate produced some discrepancies in the results to those that he had published previously (Kusuda *et al.* 1991). So an isolate of ATCC 49156 that had been obtained directly from the American Type Culture Collection was transferred by Dr Jeremy Carson from the Department of Primary Industry and Fisheries, Mount Pleasant Laboratories to the Department of Aquaculture, University of Tasmania after AQIS permission for the transfer had been obtained. Later, cultures of *Lactococcus garvieae* originally obtained from the Laboratorum voor Microbiologie Landbouwhogeschool (LMG 8893) and *Lactococcus garvieae* from a case of bovine mastitis in Tasmania (MPL 94-4127.2) were also obtained from the same source.

As the two major sites of outbreaks of the disease in Tasmania were in trout raising facilities in the Cressy and Bridport regions (Figure 4.1.1), the search for the organism was conducted around these locations. Environmental samples from various sources, including mammalian and avian faeces, septic tanks, water and mud, were collected for culture to try to isolate streptococci or enterococci similar to *Streptococcus* sp. biovar 1.

In order to compare the ten Australian reference isolates and the type strains to the 61 unknown environmental isolates which were obtained, a series of selected tests were

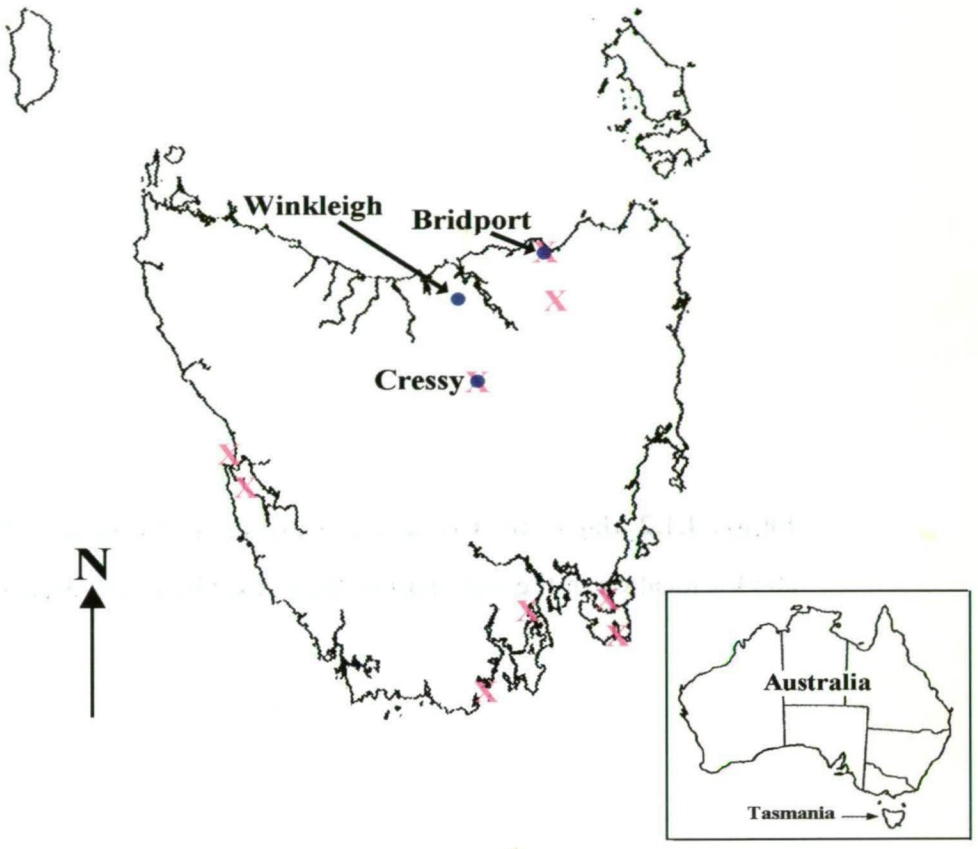
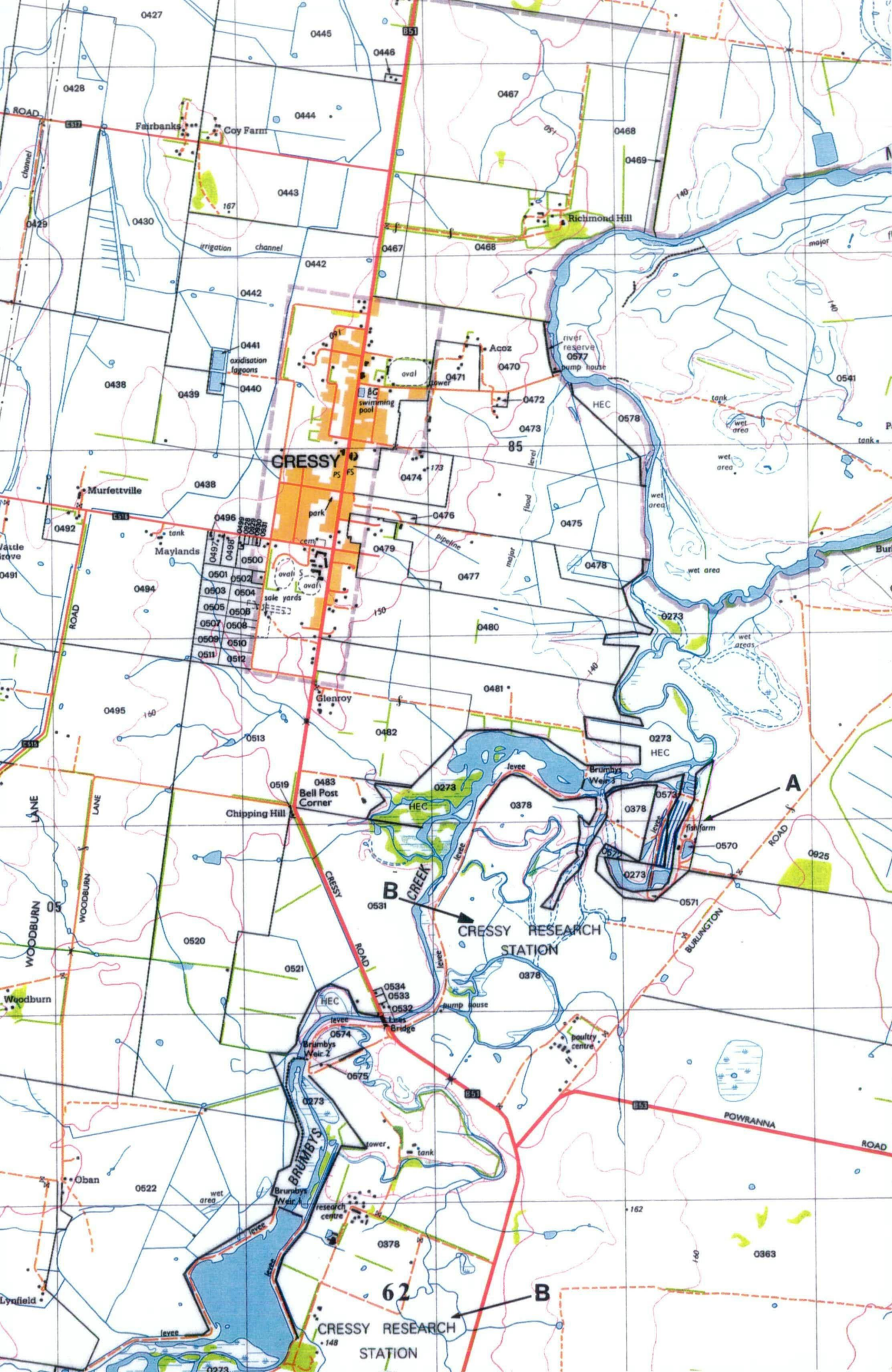



**Figure 4.1.1** Map of Tasmania showing the areas of significant outbreaks of streptococcosis in rainbow trout and the sampling sites

X Sites of outbreaks

• Sampling sites

**Figure 4.1.2** Map of the Cressy area showing the location of the fish farm **A** and the location of the main part of the Cressy Research Station **B**


CRESSY
Richmond Hill
Fairbanks
Coy Farm
Murfettville
Maylands
Glenroy
Chipping Hill
Bell Post Corner
Acoz
Oban
Lynfield
Woodburn
CRESSY RESEARCH STATION
CRESSY RESEARCH STATION
A
B
B
CREEK
BRUMBYS
CRESSY ROAD
WOODBURN ROAD
BURLINGTON ROAD
POWRANNA ROAD
WOODBURN LANE
LANE
river reserve
pump house
oxidisation lagoons
oval
tower
swimming pool
park
cem
sale yards
pipeline
flood
level
major
irrigation channel
channel
tank
wet area
wet areas
levee
HEC
Brumbys Weir 2
Brumbys Weir 1
fish farm
poultry centre
research centre
pump house
Lees Bridge
0427
0428
0429
0430
0438
0439
0440
0441
0442
0443
0444
0445
0446
0467
0468
0469
0470
0471
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0522
0531
0532
0533
0534
0541
0570
0571
0572
0573
0574
0575
0577
0578
0925
0363
0273
0378
85
62
05

**Figure 4.1.3** Map of the Bridport area showing the location of the fish farm **A**


orester Rock
ROWN LAND
0274
Beach
COASTAL
RESERVE
pipeline
Granite Point
0234
ANDERSON
BAY
35
sewage lagoons
Mermaids Beach
Old Pier Beach
old wharf piles
BRIDPORT
wet area
golf course
Mottingleys Beach
Croquet Lawn Beach
Regatta View Point
Eastmans Beach
Goftons Beach
sand pit
ROAD
gravel pits
CROWN LAND
0275
gravel pit
oval
0236
0235
QUARRY RESERVE
tanks
pump station
A
Beach
Barnbougle
coastal reserve
0272
0270
saline
coastal flats
0265
Bar Marsh
piles
fish farm
TRENT
WATER
river reserve
0271
BRIDPORT
gravel pit
pipeline
B82
0805
0868
0867
0875
training track
0876
0874
0873
0872
major flood level
WATERHOUSE
Brid River Reserve
Riverleigh
sale yards
1220
wet area
0869
gravel pit
0865
0871
0864
RIVER
ROAD
tank
BRIDPORT
Ponderosa
0870
wet area
rifle range
0877
landing ground
BRIDPORT
Shanty
tank
20
0800
medium forest with dense low vegetation
63

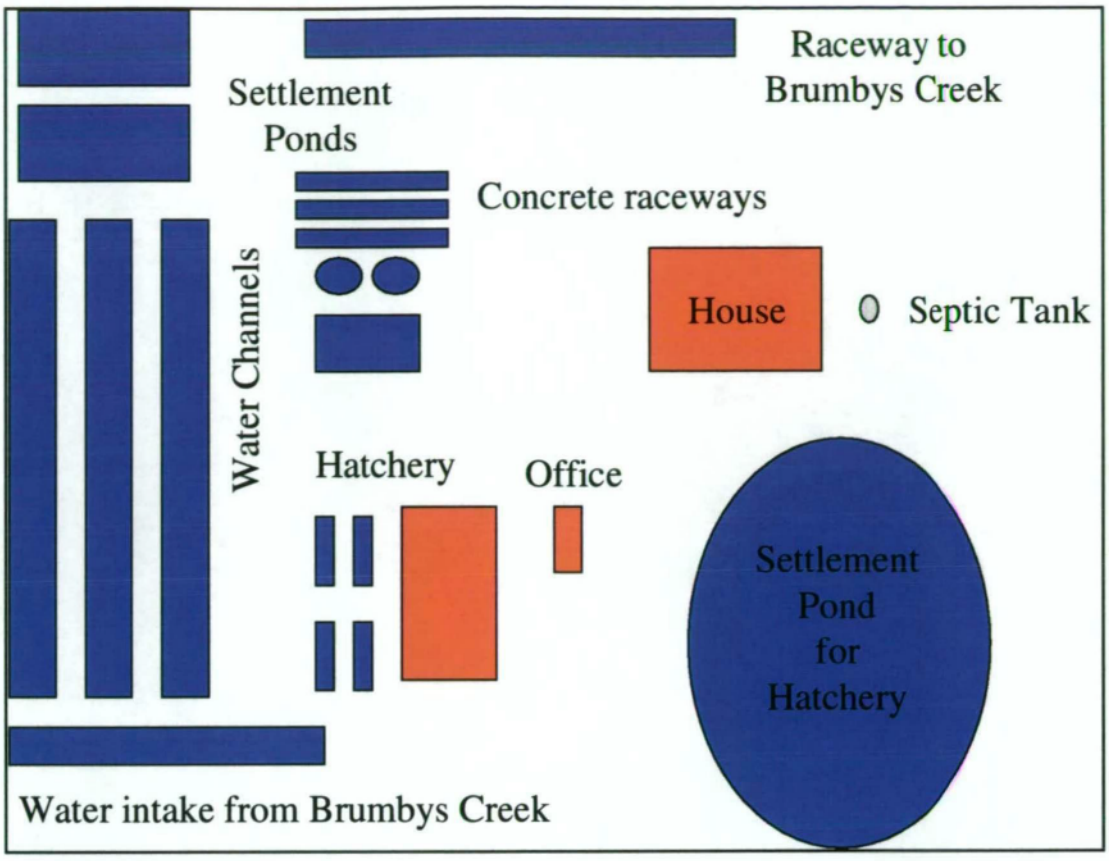



**Figure 4.1.4** Layout of the fish farm at Cressy

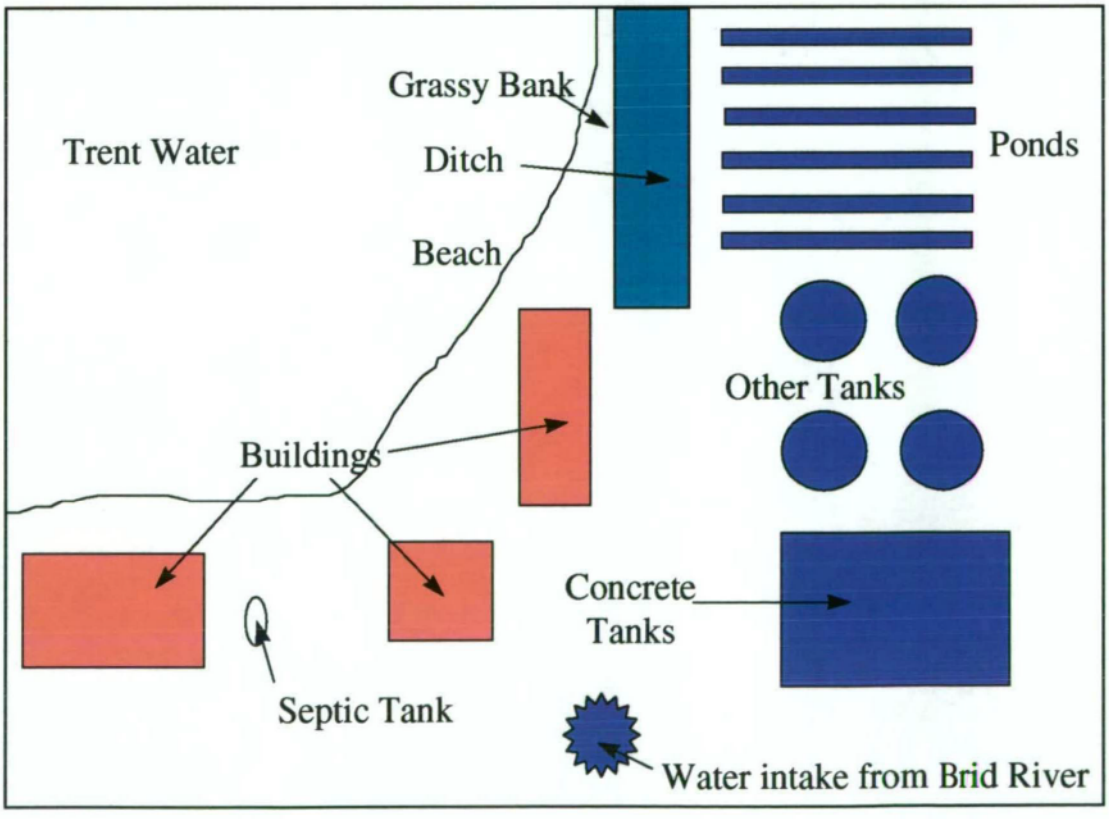



**Figure 4.1.5** Layout of the fish farm at Bridport

**Figure 4.1.6** Large ponds used for grow out at Bridport

**Figure 4.1.7** Ditch draining from farm at Bridport

**Figure 4.1.8** Sheep grazing on the grassy foreshore at Bridport

**Figure 4.1.9** Pigs at Broomby's piggery

performed on all the isolates. These tests included examination of colonial morphology, growth under different conditions and a range of selected biochemical tests. The organisms were also tested for Lancefield group D antigen, agglutination with antisera to the Australian reference isolates and antibiotic sensitivity.

The protocol set up for the characterisation of the isolates was initially based on tables in the Ninth Edition of Bergey's Manual of Determinative Bacteriology (Holt *et al.* 1994) and was later modified to include other references for the characterisation of these organisms. The other references were Cowan and Steel's Manual for the identification of Medical Bacteria (Barrow & Feltham, 1993) and API 20S and rapid ID 32 STREP™ data sheets(BioMerieux Vitek Australia Pty. Ltd. Melbourne). Isolates that were found to be similar to the type strain by individual tube-test methods using this protocol were confirmed using the commercial multi-test system rapid ID32 STREP (supplied by BioMerieux Vitek Australia Pty. Ltd. Melbourne).

### 4.1.1 Collection of samples

#### 4.1.1.1 Description of the sites of collection

The three locations where samples were collected are indicated on the map in Figure 4.1.1. The sampling took place during the summer months of 1994 and 1995. The first site for sampling was the Cressy Research Station (Figure 4.1.2), which is a farm of 1500 acres located on the banks of Brumby's Creek. This is part of the tailrace for the Poatina Power Station, upstream from Sevrup Fish Farm (Figure 4.1.2). During the period when the disease was prevalent in the fish nearby, there was a large piggery on the property. However, the piggery at Cressy was closed down in 1989 and, as there was no longer an existing piggery in the area, the property of Alan Broomby, at Winkleigh (Figure 4.1.1), where a large piggery and a chicken shed were located, was sampled.

The Sevrup fish farm is located about 1km downstream from the Cressy Research Station and consists of a large hatchery and several large grow-out ponds. There is caretaker accommodation with a septic tank on the property. The fish farm draws its water from the tailrace of the Poatina Power Station and expels further downstream (Figure 4.1.4) .

At Bridport, Purves Fisheries is located on the tidal reaches of Trent Water (Figure 4.1.3) and consists of a number of concrete tanks containing the smaller fish and six large ponds (Figure 4.1.6) , four of which are mud bottomed, for fattening rainbow trout (the hatchery is at Springfield). There are several sheds on the property, including one which has a septic tank attached. This shed is used for processing the fish (Figure 4.1.5). The farm draws its water from the non-tidal reaches of the Brid River and expels into Trent Water via a large ditch (Figure 4.1.7). Sheep (Figure 4.1.8) and wallabies graze along the grassy foreshore between the fish farm and the water. Pelicans are also commonly found nearby.

#### 4.1.1.2 Methodology of collection

Cattle and sheep faecal samples were collected into sterile plastic faecal containers (Johns Scientific ®) from the paddocks at Cressy Research Station (Department of Primary Industries). Six fresh cow pats and six fresh sheep droppings were gathered from different locations on the station. Chicken guano (6) and pig faeces (7) were similarly collected into the sterile containers from several different locations on the Winkleigh property, including indoor piggery, outdoor pigsties (Figure 4.9) and a large effluent pond draining from the pig section and also guano from the chicken house. Dead fish and faecal samples of mammalian and avian fauna were gathered at Bridport, whilst mud and water samples were collected at both Bridport and Cressy. Samples from septic tanks on the Sevrup (Cressy) and Bridport properties were collected using a device consisting of a soft plastic receptacle attached to a long length of silicon tubing. The tubing was inserted into the inspection hole at the top of the septic tank and the surface layer of sewage drawn into the receptacle by suction. The sample of platypus faeces was provided by Joanne Connolly from platypus sampled in the Deloraine area during her research into the fungus causing platypus ulcerative dermatitis.

### 4.1.2 Isolation of bacteria

#### 4.1.2.1 Processing of samples

Samples were bought to the laboratory and each one processed similarly, depending on the consistency of the original sample. If the sample was liquid, as for example cow pats or water from septic and grow-out tanks, then approximately 100µL was

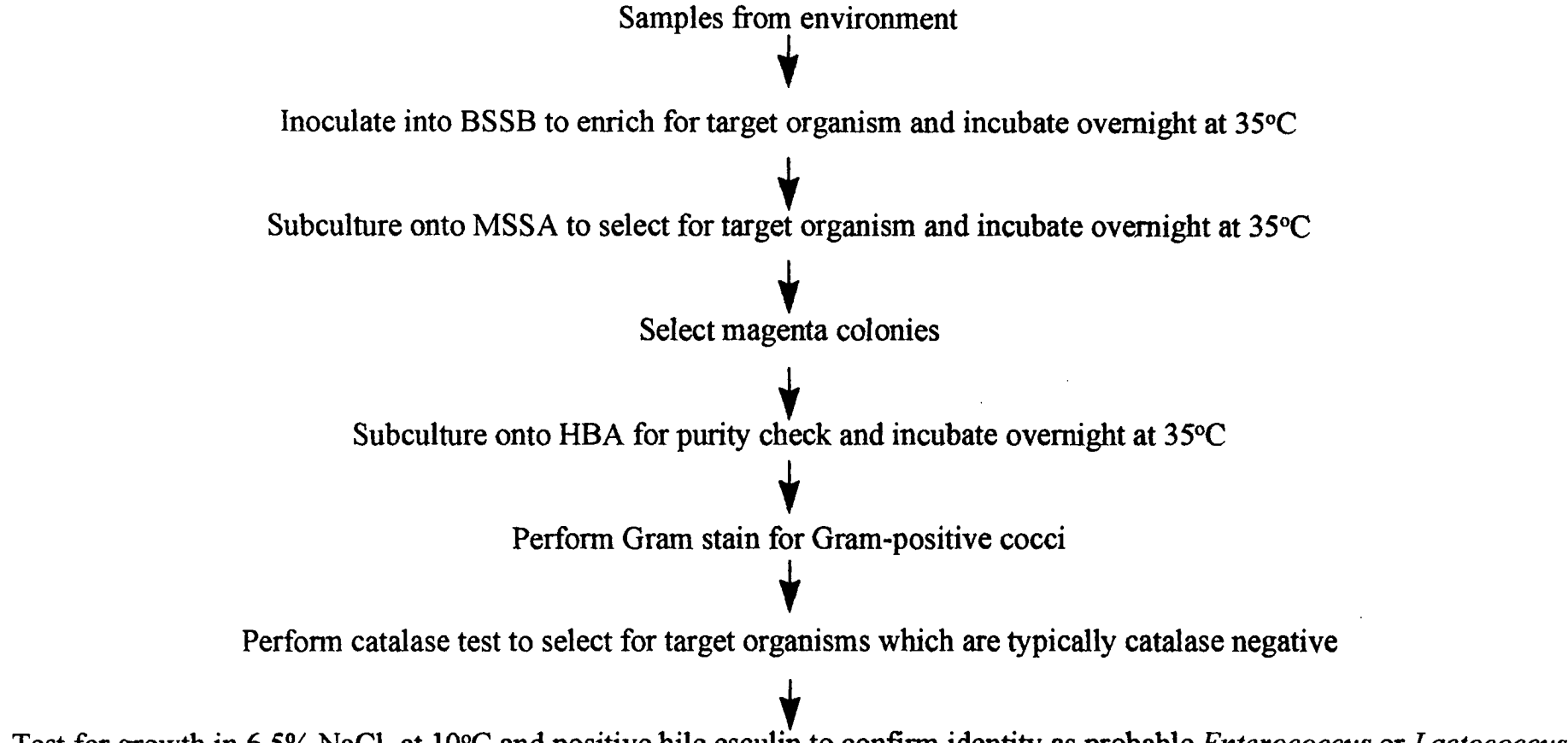



**Figure 4.1.10** Summary of the isolation and preliminary identification procedure

diluted in 2.5mL of sterile saline to make a suspension. If the sample was more formed, for example sheep faeces, then approximately 100mg of the sample was emulsified into 2.5mL of sterile saline.

A 10μL loopful of this suspension was then plated directly MSSA and 0.5mL of the suspension was inoculated into 10mL of BSSB. Both cultures were incubated aerobically overnight at 35°C. Plate cultures were examined for any growth at 18h & 48h. Colonies displaying typical morphological characteristics were subcultured onto horse blood agar (HBA) and incubated overnight. Broth cultures were subcultured onto MSSA and HBA and incubated aerobically overnight at 35°C. Plates were examined for typical colonies.

### 4.1.3 Selection of isolates using special tests

Fundamental tests of Gram stain and for catalase activity were performed first to confirm that the organism was a "streptococcus". Then, based on the results of tests performed on the Australian isolates of *Streptococcus* sp. biovar 1 (Carson & Munday, 1990; Carson *et al.* 1993), along with the information on the growth characteristics of *Enterococcus seriolicida* YT3 (Kusuda *et al.* 1991), a selection criterion was formulated for the further testing of likely organisms. As all of the isolates of *Streptococcus* sp. biovar 1 and *Enterococcus seriolicida* grew in 6.5% NaCl, at 10°C and hydrolysed esculin in the presence of 40% bile, these tests were selected for further evaluation of the isolates. The isolation of the bacteria and the selection of isolates using special tests are summarised in Figure 4.1.10. The methods for these tests are contained in the main characterisation sections 4.1.5, 4.1.6, 4.1.7

### 4.1.4 Storage of selected isolates

For the maintenance of stock cultures, isolates were plated onto 10% HBA and incubated at 35°C in air overnight or, if necessary, for 48h. The plates were examined for the presence of a pure growth of the typical colony and then the plates stored in plastic bags at 4°C for a maximum of 6 weeks, at which time fresh subcultures were prepared. This method ensured that there was always growth on solid media for any serological or biochemical test that was to be performed. As the samples were

selected they were given a simple 2 character code number, for example A1 to A9, B1 to B9 etc. and then stored at -70°C for future testing. Tests selected for the further testing of the isolates, including those already performed for the selection procedure are summarised in Table 4.1.2.

### 4.1.5 Morphological characteristics

Colony morphology, including type of haemolysis and the presence of any pigment of the isolates was examined for on the horse blood agar (HBA) plate. Microscopical morphology was determined by Gram staining.

#### 4.1.5.1 Haemolysis

Colonies of the overnight culture were examined for their haemolytic properties, initially on sheep blood agar (SBA) and then later, on horse blood agar (HBA) (Appendix A7). These media were chosen for their high performance in exhibiting haemolysis. To describe haemolytic activity, alpha-haemolysis is a zone of partial haemolysis or green discolouration in the agar immediately surrounding the colony whilst the red blood cells remain intact. With beta-haemolysis, a distinct zone of total haemolysis of the red blood cells occurs, creating a clear, colourless ring immediately surrounding the colony and the red blood cell membrane is destroyed. (Barrow & Feltham, 1993; Balows *et al.* 1991).

#### 4.1.5.2 Pigment

Since any pigment may take a longer to period develop, colonies were examined daily for up to 3d. Colonies may be described as being opaque, grey, white or yellow. Yellow pigmentation is a significant characteristic of some of the species (Holt *et al.* 1994).

#### 4.1.5.3 Gram stain

Gram stain was performed using Lillie's modification of Gram's original method (Appendix B2) employing crystal violet, Jensen's iodine, acetone/alcohol and dilute carbol fuchsin followed by examination of the slide under oil immersion (x1000).

### 4.1.6 Physiological characteristics

#### 4.1.6.1 Growth at 10°C and 45°C

Growth temperatures are especially useful for differentiating enterococci (growth at both 10°C and 45°C ) from lactococci (growth at 10°C but not 45°C) (Balows *et al.* 1991). The tests were performed at 10°C and 45°C in Oxoid brain heart infusion broth (CM225B) containing glucose for fermentation and bromocresol purple as an indicator (Appendix B3), by inoculating 4mL aliquots with a drop of 18 to 24h broth culture and incubating at 10°C in a refrigerated waterbath and at 45°C in a heated waterbath and examining for growth for up to 7d (Balows *et al.* 1991). The broths were chilled or warmed prior to inoculation and placed at the appropriate temperature within 10min of inoculation. When the tubes were inspected for growth during the incubation period they were returned to the proper temperature without being allowed to warm or cool. A positive result was recorded when the broth changed colour from purple to yellow and/or turbidity was visible in the medium.

#### 4.1.6.2 Salt tolerance

Enterococci can withstand a higher salt concentration than non-enterococci (Holt et al. 1994). Tolerance to salt was demonstrated by preparing Oxoid brain heart infusion broth (CM225B) containing 6.5% sodium chloride also containing glucose for fermentation and bromocresol purple as an indicator (Appendix B4). Cultures were inoculated, incubated at 35°C and examined for growth after 48h incubation. A positive result was recorded when the broth changed colour from purple to yellow and/or turbidity was visible in the medium.

### 4.1.7 Biochemical characteristics

#### 4.1.7.1 Catalase test

The catalase test is used to separate Gram-positive cocci into staphylococci which are catalase positive and streptococci which are catalase negative. The test employs hydrogen peroxide which, when mixed with a catalase-positive organism decomposes into water and oxygen and gas bubbles are seen (Washington, 1981).

#### 4.1.7.2 Bile esculin hydrolysis

Enterococci can grow in the presence of 40% bile and subsequently hydrolyse esculin to esculetin and glucose. Esculetin diffuses into the agar and combines with ferric citrate in the medium to give a black complex (Washington, 1981). The reactions involved can be represented by the following equation:

$$\text{Esculin} \xrightarrow{\text{Bacterial enzyme}} \text{esculetin} + \text{glucose}$$

$$\text{Esculetin} + Fe^{3+} \longrightarrow \text{black complex}$$

Bile esculin hydrolysis was tested for by inoculating Oxoid bile esculin agar (CM888B) plates, incubating overnight aerobically at 35 °C. and examining for the presence of a zone of blackening around the inoculum (Appendix B5).

#### 4.1.7.3 Arginine hydrolysis

Some strains of enterococci and other similar organisms are capable of hydrolysing arginine (Balows *et al.* 1991). Arginine hydrolysis was measured by inoculating an arginine broth with culture and incubating at 35°C for 24-48h aerobically. Hydrolysis was indicated by the formation of a brown colour after the addition of a few drops of Nessler reagent (Appendix B6).

#### 4.1.7.4 Hippurate hydrolysis

The principle of the test is the hydrolysis of sodium hippurate by hippuricase to sodium benzoate and glycine (Washington, 1981)

$$\text{Sodium hippurate} \xrightarrow{\text{hippuricase}} \text{sodium benzoate} + \text{glycine}$$

Subsequent addition of ninhydrin results in the oxidative deamination of the α-amino group in glycine to its corresponding aldehyde, with the release of carbon dioxide, ammonia and hydrinantin, the reduced form of ninhydrin. Ammonia reacts with the residual ninhydrin and hydrinantin to give a purple-coloured complex (Appendix B7).

#### 4.1.7.5 Gelatin hydrolysis

Gelatin hydrolysis was tested for by inoculating an agar plate containing 0.4 % gelatin and incubating overnight at 35°C aerobically. Hydrolysis was indicated by a clear zone

around the colony on an otherwise opaque background after flooding the plate with 20 % mercuric chloride ($HgCl_2$) (Simbert & Krieg, 1994).

#### 4.1.7.6 Acetoin test

The acetoin production test, using Baird-Parkers identification system for staphylococci and micrococci, specified a 14d incubation period for the conventional tube test. However, a 2d paper disc technique has been found to be equally sensitive and specific as the original method (Davis & Hoyling, 1973). Acetoin production was determined by inoculating a $1cm^2$ area onto a plate of tryptone glucose yeast agar (Appendix B9) and incubating at 30°C for 48h. A 6mm antibiotic disc, impregnated with 10% sodium pyruvate solution, was then placed onto the growth and the plate incubated for a further 2h. The disc was then placed into the bottom of a small test tube and 2 drops of 1% α-naphthol, 1 drop of 1% creatine and 1 drop of 40% KOH solution added (in that order), the development of a pink colour within 1h indicating a positive acetoin reaction.

#### 4.1.7.7 L-Pyrrolidonyl-β-Naphthylamide (PYR) test

The principle of the L-pyrrolidonyl-β-naphthylamide (PYR) test is that when pyrrolidonyl peptidase, present in organisms such as group A streptococci, enterococci and some strains of *Lactococcus garvieae,* catalases the hydrolysis of L-pyrrolidonyl-β-naphthylamide, it liberates free pyrrolidone carboxylic acid and β-naphthylamine (Bosley *et al.*1983). The reaction is detected by the addition of the specific indicator, *N,N*-dimethyl-amino-cinnameldehyde, which results in a red colour with a positive reaction (Appendix B10).

#### 4.1.7.8 Carbohydrate fermentation

Carbohydrate broths were prepared using a modification of the method in Barrow & Feltham (1993) for broth sugars where the indicators suggested were substituted by bromocresol purple (Appendix B11). The carbohydrates listed in Table 4.1.2 were sterilised by membrane filtration and added to the broth at a concentration of 0.5%. These were then inoculated with culture and incubated aerobically for 24-48h at 35°C.

Fermentation was indicated by a change in colour from purple to yellow. The lids of the tubes of each carbohydrate were colour coded for ease of recognition.

**Table 4.1.1 List of carbohydrates used in the characterisation of the isolates**

| | |
|---|---|
| Arabinose | Ribose |
| Lactose | Sorbitol |
| Mannitol | Starch |
| Raffinose | Trehalose |

### 4.1.8 Immunological techniques

#### 4.1.8.1 Whole-cell agglutination

This is a quick and easy technique which can provide some useful data. A loopful of bacterial culture is emulsified in a drop of physiological saline on a glass slide. A drop of specific antiserum is added and mixed thoroughly with the bacterial suspension with a sterile loop. The slide is then rocked gently for about 2min and examined for agglutination or clumping of the cells (Austin & Austin, 1993). The antisera used in this experiment were sheep anti-*Streptococcus* antisera 1 prepared by M. Akhlaghi (Akhlaghi *et al.* 1996).

#### 4.1.8.2 Lancefield group antigen

The principle of the Lancefield group antigen test involves the extraction of specific carbohydrate antigens from different strains of streptococci and agglutinating them with blue latex particles (Oxoid DR 589) coated with group-specific antibody. These particles agglutinate in the presence of homologous antigen, forming a lattice which is clearly visible as a positive result. Using the enzymatic extraction technique the test can be interpreted almost immediately and the antigen yield improved especially for Group D streptococci and enterococci.

The method involved emulsifying 2 to 5 isolated colonies in 0.4mL of Oxoid extraction enzyme (DR593). This was then incubated at 37°C for 10min with a thorough mixing after 5min. The latex reagents were mixed thoroughly and one drop dispensed onto an appropriately labelled circle on the disposable reaction card (Oxoid DR500). One drop of the sample extract was then added to the latex drop with a

Pasteur pipette and mixed with a wooden stick, spreading the contents of the circle to cover the entire area. The card was gently rocked in a circular motion for up to 1min and observed for agglutination. A positive result was clumping of the blue latex particles, visible to the naked eye.

### 4.1.9 Antibiotic sensitivity

Some strains of enterococci and lactococci have characteristic antibiograms and, therefore, sensitivity or resistance to a certain antibiotic can be used as part of the characterisation profile (Facklam *et al.* 1995). The antibiotics chosen for this experiment were selected using recent literature on enterococcal and lactococcal antibiograms. The method used was adapted from the calibrated dichotomous sensitivity (CDS) testing method (Appendix B12) and the antibiotics used were amoxicillin, penicillin, clindamycin, erythromycin, cephalothin, nitrofurantoin and vancomycin.

### 4.1.10 Analysis of the test results

The results of all the tests were entered into a table as per Table 4.1.2.
The tests performed to characterise the isolates were built into a table and the results of tests on species of *Lactococcus* and *Enterococcus* from the literature were added as a means of characterising the unknown strains. The data for this table was drawn from three major sources, published papers by workers in taxonomy, commercial test system identification tables and books on taxonomy. The source of most of this data is summarised in Table 4.1.3. The characterisation of the lactococci and the enterococci from the some of the resources are shown in tables in the Appendix section (C1.1 to C1.4) and were used as a template to try to classify the unknown isolates into a known species.

A flow chart (Figure 4.1.11) was designed so that the 76 isolates could be separated into smaller groups. The tests chosen for the flow chart were growth at 10°C to separate *Streptococcus* from *Lactococcus* and *Enterococcus.* The PYR test was then included to differentiate between *Lactococcus* sp. (except *Lactococcus garvieae*) and *Enterococcus* sp.. The next characteristic included was the Lancefield group D antigen test to eliminate *Lactococcus* and for preliminary differentiation of the strains

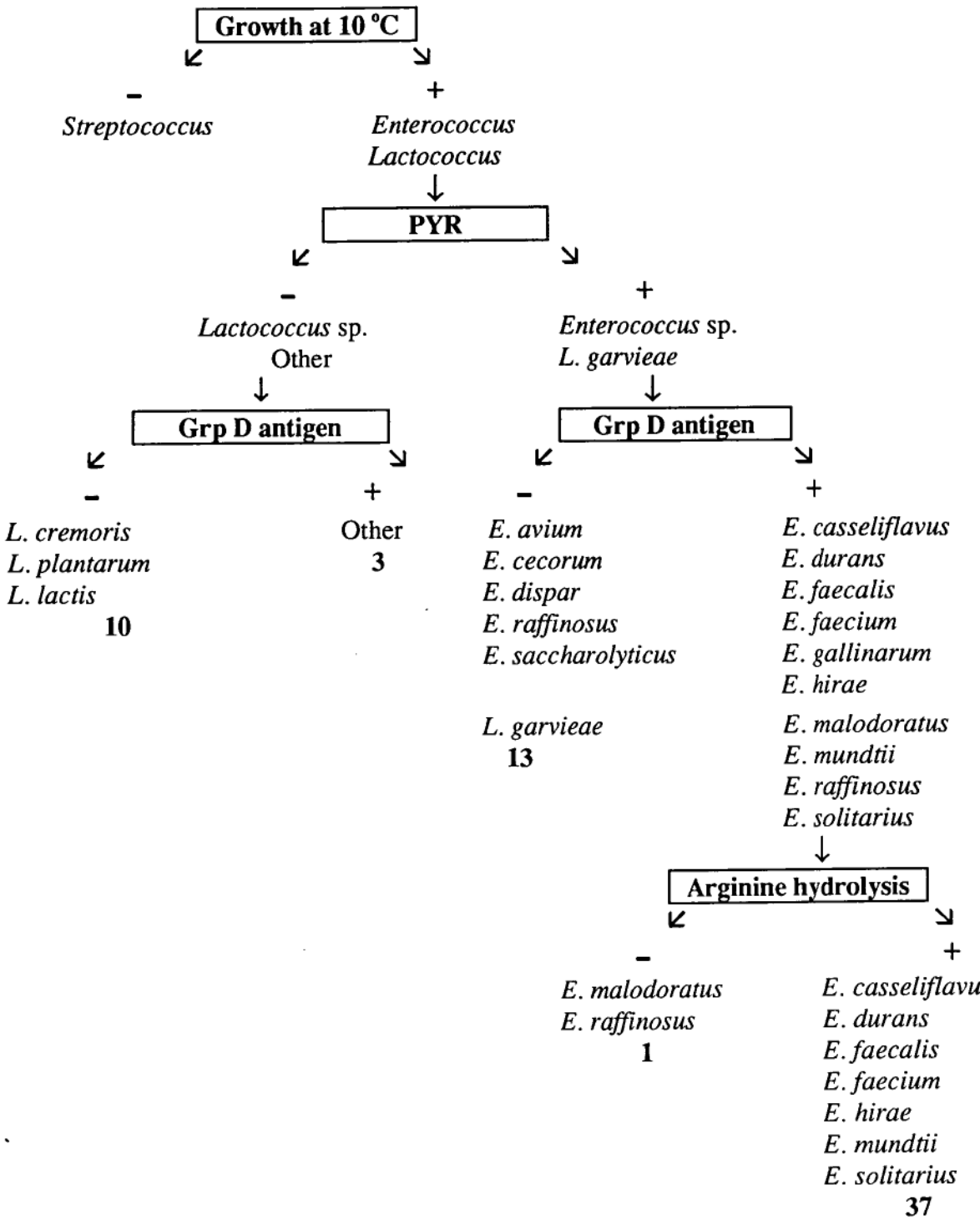



**Figure 4.1.11** Flow chart for preliminary characterisation of isolates

of *Enterococcus* sp. that show a negative Lancefield group D antigen result. Arginine

**Table 4.1.2 Example of the table used for entering characterisation results**

| TEST | RESULT |
|---|---|
| Haemolysis | α/β/γ |
| Yellow pigment | ± |
| Growth at: | |
| 10°C | ± |
| 45°C | ± |
| Growth in: | |
| 6.5% NaCl | ± |
| Bile Esculin | ± |
| Arginine hydrolysis | ± |
| Hippurate hydrolysis | ± |
| Gelatin | ± |
| VP/ acetoin reaction | ± |
| PYR | ± |
| Fermentation of: | |
| Arabinose | a/- |
| Lactose | a/- |
| Mannitol | a/- |
| Raffinose | a/- |
| Ribose | a/- |
| Sorbitol | a/- |
| Starch | a/- |
| Trehalose | a/- |
| Agglutination | pos/neg/w |
| Lancefield group | D/Not D |
| Sensitivity to: | |
| Amoxicillin 10 | S/R |
| Penicillin 10 | S/R |
| Clindamycin 2 | S/R |
| Erythromycin 15 | S/R |
| Cephalothin 30 | S/R |
| Nitrofurantoin 100 | S/R |
| Vancomycin 30 | S/R |

**Table 4.1.3 Summary of the source of data used to characterise isolates in this experiment**

| Type of Reference | Source | Number of Enterococci | Number of Lactococci |
|---|---|---|---|
| Authors | Collins M.D. | | 1 |
| | Schleiffer | | 2 |
| Multi-test systems | API 20S | 9 | 3 |
| | rapid ID 32 STREP | 8 | 4 |
| Books | Cowan & Steel | 7 | 1 |
| | Bergey's Manual | 16 | 0 |

hydrolysis was finally included in the program to further characterise the lactococci and some of the enterococci.

### 4.1.11 Multi-test system

The multi-test system chosen was the rapid ID 32 STREP™ kit (bioMériuex Vitek) which can identify a range of organisms including 37 species of *Streptococcus*, 8 species of *Enterococcus* and 4 species of *Lactococcus* including *Lactococcus garvieae*. The kit does not include identification of *Enterococcus seriolicida*. The organisms selected for this additional test were the Australian isolates, *Enterococcus seriolicida* (ATCC 49156), *Enterococcus seriolicida* YT3, *Lactococcus garvieae* (LMG 8893) and *Lactococcus garvieae* (MPL 94-4127.2), and isolates that did not possess the Lancefield's Group D antigen and/or which agglutinated with the antisera. The test was performed as per the instructions on the package insert.

**Table 4.1.4 Summary of survey isolates tested with the multi test system, rapid ID 32 STREP (section 4.1.11)**

| Not Group D | Agglutination |
|---|---|
| UT1C | w |
| UT9C | - |
| UT1D | - |
| UT2D | + |
| UT6D | w |
| UT7D | - |
| UT4E | + |
| UT7E | + |
| UT8E | w |
| UT9E | - |
| UT7F | - |
| UT8F | - |
| UT9F | - |
| UT2G | + |
| UT9H | w |
| UT1I | - |

## Results

### 4.1.1 Collection of samples

Approximately 100 different sample types were collected from the sites as follows:-

**Cressy**

| | | | | | |
|---|---|---|---|---|---|
| Septic tank | 1 | Inflow | 4 | Outflow | 6 |
| Mud | 6 | Tanks | 6 | Ponds | 6 |

| | | | | | |
|---|---|---|---|---|---|
| Cattle | 6 | Sheep | 6 | | |
| **Bridport** | | | | | |
| Septic tank | 2 | Inflow | 4 | Outflow | 6 |
| Mud | 6 | Tank | 6 | Ponds | 6 |
| Fish | 5 | Sheep | 4 | Pelican | 1 |
| Wallaby | 2 | | | | |
| **Winkleigh** | | | | | |
| Pig | 7 | Chicken | 6 | | |
| **Deloraine** | | | | | |
| Platypus | 1 | | | | |

### 4.1.2 Isolation of bacteria

#### 4.1.2.1 Processing of the samples

The samples were processed by the protocol summarised in Figure 4.1.10

### 4.1.3 Selection of isolates

Using the selection protocol, 61 isolates were selected for further testing. The number of isolates from the different types of sample and their collection locations are summarised in Table 4.1.5. The basic tests used to eliminate organisms that were not similar to the target organism were selected from numerous reports of the characteristics of organisms causing streptococcal fish disease including those summarised in Table 4.1.6.

**Table 4.1.5 Number of isolates from samples collected from each of the different locations**

| Location | Sample Type | Number of isolates | Location | Sample Type | Number of isolates |
|---|---|---|---|---|---|
| Cressy | Septic tank | 4 | Bridport | Outflow | 3 |
| Cressy | Inflow | 2 | Bridport | Mud | 2 |
| Cressy | Outflow | 4 | Bridport | Tank | 5 |
| Cressy | Mud | 2 | Bridport | Pond | 5 |
| Cressy | Tank | 1 | Bridport | Fish | 5 |
| Cressy | Pond | 3 | Bridport | Sheep | 3 |
| Cressy | Cattle | 3 | Bridport | Pelican | 1 |
| Cressy | Sheep | 2 | Bridport | Wallaby | 2 |
| Bridport | Septic tank | 2 | Winkleigh | Pig | 5 |
| Bridport | Inflow | 6 | Winkleigh | Chicken | 1 |
| | | | Deloraine | Platypus | 1 |

The total number of isolates tested was 76, including the 10 Australian isolates of *Streptococcus* sp. biovar 1, the 4 type strains which include 2 isolates of *Enterococcus seriolicida* (ATCC 49156), 1 isolate of *Enterococcus seriolicida* YT3, *Lactococcus garvieae* (LMG 8893) and the bovine isolate of *Lactococcus garvieae* (MPL 94-

**Table 4.1.6 Summary of the basic test results for organisms causing streptococcal disease in fish.**

| | Foo *et al* 1985 | Bragg & Broere 1986 | Kusuda *et al* 1991 | Ceschia *et al* 1992 | Carson *et al* 1993 | Toranzo *et al* 1994a |
|---|---|---|---|---|---|---|
| Gram stain | + | + | + | + | + | + |
| Catalase | - | - | - | - | - | - |
| Growth at: | | | | | | |
| 10°C | + | + | ND | ND | + | + |
| Growth in: | | | | | | |
| 6.5% NaCl | + | - | + | ND | + | + |
| Bile esculin | + | + | ND | + | + | + |

ND = no data

4127.2) and the 61 unknown isolates. The full results of their characterisation for all these isolates are tabled in Appendix section tables (see Tables C2.1 to C2.9 in the appendix section). Table 4.1.7 summarises the different isolates from the survey including the Australian isolates, the type strains, the bovine isolate of *Lactococcus garvieae* and the 61 unknown isolates. Table 4.1.8 summarises all the isolates and their identification. Table 4.1.9 summarises the characterisation results of two of the Australian isolates of *Streptococcus* sp. biovar 1, *Enterococcus seriolicida* (ATCC 49156), *Lactococcus garvieae* (LMG 8893) and the bovine isolate of *Lactococcus garvieae* (MPL 94-4127.2). Of the 10 Australian isolates of previously *Streptococcus* sp. biovar 1 there were 2 profiles, and therefore 2 strains, by the conservative method, with the only difference being the fermentation or non-fermentation of lactose.

### 4.1.5 Morphological characteristics

Overall, morphologically, the colonies were smooth, translucent grey to white and with an entire edge with or without a surrounding zone of haemolysis. There was no discernible difference between the haemolysis seen on SBA, which was used in the initial part of the experiment, to that of the haemolysis on HBA. These findings are typical of the morphology of the species of catalase negative, Gram-positive cocci.

Some of the isolates displayed a yellow pigment. All the isolates were Gram-positive cocci either in chains or pairs. The results of the haemolysis and pigmentation of the isolates are summarised in Tables C2.1 to C2.9 in Appendix C. All of the organisms were Gram-positive cocci.

### 4.1.6 Physiological characteristics

Of the 74 isolates, all except 1 grew at 10°C (98.6% positive) while 29 isolates failed to grow at 45 °C (39.2%) or 60.8% positive. The isolate that failed to grow at 10 °C also failed to grow at 45°C and was therefore not recognised as an enterococcus or as a lactococcus. The results are summarised in Tables C2.1 to C2.9 in Appendix C.

### 4.1.7 Biochemical characteristics

All of the organisms were catalase negative and the remainder of the test results are summarised in Tables C2.1 to C2.9 in Appendix C.

### 4.1.8 Immunological techniques

The whole cell agglutination test gave weak or positive results for 23 of the 76 isolates and the Lancefield group antigen test found that 45 of the 76 isolates were positive to Group D antigen. The results are summarised in Tables C2.1 to C2.9 in Appendix C.

### 4.1.9 Antibiotic sensitivity

Sensitivity testing showed sensitivity of 89% to amoxicillin, 86% to penicillin, 15% to clindamycin, 75% to erythromycin, 14% to cephalithin, 55% to nitrofurantoin and 97% to vancomycin. The results are summarised Tables C2.1 to C2.9 in Appendix C.

### 4.1.10 Analysis of the test results

The results of the analysis of the tests are summarised in Table 4.1.7 and 4.1.8

### 4.1.11 Multi-test system

The results of the identification of the isolates using the rapid ID 32 STREP system the Australian isolates gave profile numbers of either 30333001010 or 30323001010, *Enterococcus seriolicida* YT3 30233001010 whilst *Enterococcus*

*seriolicida* (ATCC 49156) 30323001010, *Lactococcus garvieae* (MPL 94-4127.2) and *Lactococcus garvieae* (LMG 8893) all gave the profile number of 30333001010. Both of these profile numbers showed a 99.5% accuracy rating for identification as *Lactococcus garvieae* and the only test against both these profile numbers was methyl-β-D-glucopyranos (MBDG) which is usually positive and against profile number 30333001010 was lactose which is usually negative according to this system. None of the other organisms tested (see Table 4.1.4) by this method were identified as *Lactococcus garvieae.*

The results of all the organisms tested by either or both methods may be found in Table 4.1.8. Table 4.1.9 summarises the characterisation results of the Australian isolates of *Streptococcus* sp. biovar 1, *Enterococcus seriolicida* (ATCC 49156), *Enterococcus seriolicida* YT3, *Lactococcus garvieae* (LMG 8893) and the bovine isolate of *Lactococcus garvieae.* Of the 42 organisms identified as belonging to the enterococcal genus, only 23 could be identified to a species level, and of 27 organisms identified as belonging to the lactococcal genus, none could be identified to species level other than those identified as *Lactococcus garvieae.*

**Table 4.1.7 Summary of the number of different isolates from the survey including the Australian isolates and the culture collection type strains**

| ISOLATE | TOTAL NUMBER |
|---|---|
| *Enterococcus* sp. | 23 |
| *E. faecalis* | 7 |
| *E. faecium* | 3 |
| *E. mundtii* | 4 |
| *E. casseliflavus* | 3 |
| *E. hirae* | 2 |
| *Lactococcus* sp. | 12 |
| *L. garvieae* | 15 |
| *Streptococcus* sp. | 1 |
| Unknown | 6 |

**Table 4.1.8 Summary of the identification of isolates from the survey**

| Code | Sample type | Source | Identification |
|---|---|---|---|
| MP6 | Culture | Mt Pleasant | *L. garvieae* |
| MP9 | Culture | Mt Pleasant | *L. garvieae* |
| MP16 | Culture | Mt Pleasant | *L. garvieae* |
| MP17 | Culture | Mt Pleasant | *L. garvieae* |
| MP20 | Culture | Mt Pleasant | *L. garvieae* |
| MP21 | Culture | Mt Pleasant | *L. garvieae* |
| MP28 | Culture | Mt Pleasant | *L. garvieae* |
| MP36 | Culture | Mt Pleasant | *L. garvieae* |
| MP38 | Culture | Mt Pleasant | *L. garvieae* |
| MP39 | Culture | Mt Pleasant | *L. garvieae* |
| UT2B | Culture | Kusuda | *L. garvieae* |
| UT3B | Culture | Kusuda | *L. garvieae* |
| UT4B | Sheep faeces | Cressy | *E. casseliflavus* |
| UT5B | Cattle faeces | Cressy | *E. hirae* |
| UT6B | Pig faeces | Winkleigh | *E. casseliflavus* |
| UT7B | Pig faeces | Winkleigh | *E. hirae* |
| UT8B | Pig faeces | Winkleigh | *E. faecalis* |
| UT9B | Pig faeces | Winkleigh | *E. mundtii* |
| UT1C | Pig effluent | Winkleigh | *Lactococcus* sp. |
| UT2C | Chicken faeces | Winkleigh | *E. faecium* |
| UT3C | Cattle faeces | Cressy | *E. mundtii* |
| UT4C | Mud | Cressy | *Enterococcus* sp. |
| UT5C | Mud | Cressy | *Enterococcus* sp. |
| UT6C | Septic tank | Cressy | *E. faecalis* |
| UT7C | Septic tank | Cressy | *E. mundtii* |
| UT8C | Septic tank | Cressy | *E. faecalis* |
| UT9C | Outflow | Cressy | *Lactococcus sp.* |
| UT1D | Cattle faeces | Cressy | *Lactococcus* sp. |
| UT2D | Septic tank | Cressy | *Lactococcus* sp. |
| UT3D | Outflow | Cressy | unknown |
| UT4D | Outflow | Cressy | *Enterococcus* sp. |
| UT5D | Mud | Bridport | *Enterococcus* sp. |
| UT6D | Septic tank | Bridport | unknown |
| UT7D | Septic tank | Bridport | *Lactococcus sp.* |
| UT8D | Outflow | Bridport | *E. faecalis* |
| UT9D | Inflow | Bridport | *Enterococcus* sp. |
| UT1E | Inflow | Bridport | *Enterococcus* sp. |
| UT2E | Sheep faeces | Bridport | *E. mundtii* |

cont.....

.....cont **Table 4.1.8**

| Code | Sample type | Source | Identification |
|---|---|---|---|
| UT3E | Sheep faeces | Bridport | *E. faecium* |
| UT4E | Pelican | Bridport | *Lactococcus sp.* |
| UT5E | Wallaby | Bridport | *E. casseliflavus* |
| UT6E | Wallaby | Bridport | *Enterococcus* sp. |
| UT7E | Tank | Bridport | *Lactococcus sp.* |
| UT8E | Tank | Bridport | *Lactococcus* sp. |
| UT9E | Sheep | Bridport | *Lactococcus* sp. |
| UT1F | Pond | Bridport | *Enterococcus* sp. |
| UT2F | Fish kidney | Bridport | unknown |
| UT3F | Fish kidney | Bridport | unknown |
| UT4F | Fish gut | Bridport | *E. faecalis* |
| UT5F | Pond | Cressy | unknown |
| UT6F | Pond | Cressy | *Enterococcus* sp. |
| UT7F | Pond | Cressy | *Enterococcus* sp. |
| UT8F | Septic tank | Cressy | *Lactococcus* sp. |
| UT9F | Platypus faeces | Deloraine | *Lactococcus* sp. |
| UT1G | Fish gut | Bridport | *Enterococcus* sp. |
| UT2G | Fish gut | Bridport | *Lactococcus* sp. |
| UT3G | In flow | Bridport | *Enterococcus* sp. |
| UT4G | In flow | Bridport | *E. faecalis* |
| UT5G | In flow | Bridport | *Enterococcus* sp. |
| UT6G | Tank | Bridport | *E. faecium* |
| UT7G | Tank | Bridport | *Enterococcus* sp. |
| UT8G | Tank | Bridport | *E. faecalis* |
| UT9G | Pond | Bridport | *Enterococcus* sp. |
| UT1H | Pond | Bridport | *Enterococcus* sp. |
| UT2H | Pond | Bridport | *Enterococcus* sp. |
| UT3H | Out flow | Bridport | *Enterococcus* sp. |
| UT4H | Out flow | Bridport | *Enterococcus* sp. |
| UT5H | In flow | Cressy | *Enterococcus* sp. |
| UT6H | In flow | Cressy | *Enterococcus* sp. |
| UT7H | Tank | Cressy | *Enterococcus* sp. |
| UT8H | Outflow | Cressy | *Enterococcus* sp. |
| UT9H | In flow | Bridport | unknown |
| UT1I | Pond | Bridport | *Streptococcus* sp. |
| UT2I | Culture | Mt Pleasant | *L. garvieae* |
| MPLG1 | Culture | Mt Pleasant | *L garvieae* |
| MPLG2 | Culture | Mt Pleasant | *L garvieae* |

**Table 4.1.9 Characterisation results of the Australian isolates of *Streptococcus* sp. biovar 1, *Enterococcus seriolicida* (ATCC 49156), *Enterococcus seriolicida* YT3, *Lactococcus garvieae* (LMG 8893) and the bovine isolate of *Lactococcus garvieae***

| | *Streptococcus* sp. biovar 1 MP9 | *Streptococcus* sp. biovar 1 MP16 | *Enterococcus seriolicida* YT3 | *Enterococcus seriolicida* ATCC 49156 | *Lactococcus garvieae* LMG 8893 | *Lactococcus garvieae* (bovine strain) |
|---|---|---|---|---|---|---|
| Haemolysis | α | α | α | α | α | α |
| Yellow pigment | - | - | - | - | - | - |
| Growth at: | | | | | | |
| 10°C | + | + | + | + | + | + |
| 45°C | - | - | - | - | - | - |
| Growth in: | | | | | | |
| 6.5% NaCl | + | + | + | + | + | + |
| Bile Esculin | + | + | + | + | + | - |
| Arginine hydrolysis | + | + | + | + | + | + |
| Hippurate hydrolysis | - | - | - | - | - | - |
| Gelatin | - | - | - | - | - | - |
| VP/ acetoin reaction | + | + | + | + | + | + |
| PYR | + | + | + | + | + | + |
| Arabinose | - | - | - | - | - | - |
| Lactose | - | a | - | a | a | a |
| Mannitol | a | a | a | a | a | a |
| Raffinose | - | - | - | - | - | - |
| Ribose | a | a | a | a | a | a |
| Sorbitol | - | - | - | - | - | - |
| Starch | - | - | - | - | - | - |
| Trehalose | a | a | a | a | a | a |
| Agglutination | pos | pos | pos | pos | pos | w |
| Lancefield group | Not D | Not D | Not D | Not D | Not D | Not D |
| Sensitivity to: | | | | | | |
| Amoxicillin 10 | S | S | S | S | S | S |
| Penicillin 10 | S | S | S | S | S | S |
| Clindamycin 2 | R | R | R | R | R | R |
| Erythromycin 15 | S | S | S | S | S | S |
| Cephalothin 30 | R | R | R | R | R | R |
| Nitrofurantoin 100 | R | S | R | R | R | R |
| Vancomycin 30 | S | S | S | S | S | S |
| Capsule | - | - | - | - | - | - |

**Discussion**

From these studies it became apparent that *Streptococcus* sp. biovar 1 is actually *Lactococcus garvieae* and this identification will be used from here on.

The choice of sites for the sampling was dictated by the areas of previous outbreaks of the disease. As *Streptococcus* sp. was found in mud and sea water near yellowtail culturing pens (Kitao *et al.* 1979) this type of sample was a likely choice to start with. Also there was the potential of the source of disease being the rainbow trout themselves or faecal contamination from domestic animals (cattle, sheep, pigs or chickens), wild animals (mammals or birds) or man. Therefore, samples from all these potential sources were collected for examination.

The fact that the piggery at Cressy was operational during the periods of the out breaks of disease in the fish at Sevrup and had closed down when the environmental sampling was undertaken means that the source of infection being from the pigs previously held at Cressy cannot be excluded. The type of food that was fed to the pigs may have contained milk products, although they were not fed with the skim milk from a creamery (M. Fenton, 1997 pers. comm.). The type of environment around a piggery where copious amounts of water are used to maintain a clean habitat, would suggest that there would be a greater amount of contaminated run-off from that area. Theoretically this excess run-off would then make its way to the ground water and surrounding waterways.

As there is only fattening of cattle and sheep and no milk production at the Cressy research station, the likelihood excess run-off from the cattle and sheep side of the property would probably be considerably less than that of a milking shed or the original piggery.

Phenotypic characterisation of bacteria is fraught with difficulties. In this study, there were, overall, some discrepancies between the findings of different authors on the identification of the causative organisms of streptococcosis, whether they were at the time identified as *Streptococcus* sp. biovar 1, *Enterococcus seriolicida* or *Lactococcus garvieae* or even *Streptococcus iniae* and *Streptococcus parauberis*.

Identification of bacteria using standard techniques has been undertaken for many years now. The genus *Streptococcus* has undergone major transformation in the last 15 years, with this catalase negative, Gram-positive coccus being reclassified into at least four major genera, including *Streptococcus* sp., *Enterococcus* sp., *Lactococcus* sp. and *Vagococcus* sp. including at least 30 species and sub-species of streptococci, 19 species of enterococci, and 6 species and sub-species of lactococci being named (Schleiffer & Kilpper-Bälz, 1987; Devriese *et al.*1993). Many organisms still remain unnamed, as normally only the ones that cause pathogenicity in humans and to a lesser degree other animals and plants are classified fully and with characterisation results available for reference.

In this study, most of the isolates could be classified as belonging to either the genus *Enterococcus* or *Lactococcus* and some were characterised to species level whilst a few could not be classified under either genus. Although these organisms could almost be classified into one genus, there was at least one test or reaction that contradicted the characterisation. Therefore, they did not fit into any known genus documented to date.

As *Enterococcus seriolicida* was only classified in the reference text Bergey's Manual of Determinative Bacteriology 9 (Holt *et al.* 1994) using the results that Kusuda *et al.* (1991) had determined, none of the Australian isolates of *Lactococcus garvieae* could be identified using this reference because of the discrepancies in the sorbitol reactions and growth at 45°C.

The ten Australian isolates of *Streptococcus sp.* biovar 1, the Japanese strains *Enterococcus seriolicida* (ATCC 49156) and *Enterococcus seriolicida* YT3, the *Lactococcus garvieae* (LMG 8893) and the *Lactococcus garvieae* (MPL 94-4127.2) all identified as *Lactococcus garvieae* by the rapid ID 32 STREP system. None of the 61 isolates from the environment, mostly characterised as *Enterococcus* and *Lactococcus species,* identified as *Lactococcus garvieae.*

Teixeira *et al.*(1996) (Table 1.4, Chapter one) found that the type strain *Enterococcus seriolicida* (ATCC 49156) was sorbitol negative which was in agreement with our

**Table 4.1.10** Summary of the results of cultural and biochemical tests performed on the American Type Culture Collection strain of *Enterococcus seriolicida* (ATCC 49156) by seven different authors.

| | Kusuda *et al.* 1991 | Domenéch *et al.* 1993 | Toranzo *et al.* 1994a | Schmidtke & Carson 1994 | Salati *et al.* 1996 | Teixeira *et al.* 1996 | Alim *et al.* 1996 |
|---|---|---|---|---|---|---|---|
| Haemolysis | α | | α | α | | | α |
| Yellow pigment | | | - | | | | |
| Gram stain | | | + | | | | |
| Growth at 10°C | | | + | | + | + | + |
| 45°C | + | | + | | | + | |
| Growth in: | | | | | | | + |
| 6.5% NaCl | + | | + | | + | + | |
| 0.01% TTZ | + | | | | | | |
| Bile Esculin | | | + | + | | + | |
| Arginine hydrolysis | + | + | + | + | + | + | |
| Hippurate hydrolysis | - | | - | - | - | - | |
| VP/ acetoin reaction | + | | + | + | -/+ | + | |
| PYR | | + | + | + | - | + | |
| Arabinose | | | - | - | - | - | |
| Lactose | - | + | (±) | - | - | - | |
| Mannitol | + | + | + | + | + | + | |
| Raffinose | - | - | - | - | - | - | |
| Ribose | | + | + | | + | + | |
| Sorbitol | + | - | (±) | - | - | - | |
| Starch | | - | - | - | - | | |
| Trehalose | | + | + | + | + | + | |

**Table 4.1.11** Physiological and biochemical characteristics of streptococci associated with fish disease compared to my findings

| | Foo *et al.* 1985 | Bragg & Broere 1986 | Kusuda *et al.* 1991 | Ceschia *et al.* 1992 | Carson *et al.* 1993 | Toranzo *et al.* 1994a | Teixeira *et al.* 1996 | Domenéch *et al.* 1996 | Hawkesford 1997 |
|---|---|---|---|---|---|---|---|---|---|
| Growth at: | | | | | | | | | |
| 10°C | + | + | | | + | + | + | + | + |
| 22°C | | | | + | | | | + | + |
| 37°C | | | | + | + | + | | + | + |
| 45°C | - | | + | | - | - | + | - | - |
| pH 9.6 | + | | | | | weak | | - | |
| Growth in: | | | | | | | | | |
| 4.5% NaCl | | | | | | | | + | |
| 6.5% NaCl | + | | + | | + | + | + | - | + |
| 0.01% TTZ | | | + | | | | | | + |
| Esculin | + | + | | + | + | + | + | | + |
| Haemolysis | α | | α | | | α | | | α |
| Arginine hydrolysis | | + | + | + | + | + | + | + | + |
| Hippurate hydrolysis | - | - | - | | - | + | - | +/- | - |
| VP/ acetoin reaction | | | + | + | + | + | + | + | + |
| PYR | + | | | + | + | + | + | + | + |
| Arabinose | - | - | | - | - | - | - | - | - |
| Glycerol | + | | - | | - | - | - | - | |
| Lactose | - | - | - | - | - | +/- | + | + | +/- |
| Mannitol | + | - | + | + | + | + | - | + | + |
| Melibiose | - | | - | | - | | - | - | |
| Raffinose | - | - | - | - | - | - | - | - | - |
| Ribose | + | | + | | | + | + | + | + |
| Salicin | + | + | | | + | | | + | |
| Sorbitol | - | - | + | - | - | +/- | - | + | - |
| Starch | | - | | + | - | - | | - | - |
| Sucrose | + | | - | | - | | - | | |
| Trehalose | + | + | | + | + | + | + | + | + |
| Lancefield group | | | Not D | | Not D | No group | No group | | Not D |

findings. They also found that, contrary to our findings, it was able to grow it at 45°C. With two type strains of *Lactococcus garvieae* (ATCC 43921 and SS 1290) that they tested for carbohydrate fermentation, the fermentation of lactose was the only discrepancy between the two strains. This was the case also with the Australian isolates and therefore in agreement with our findings.

Kusuda based his belief that the organism was not a *Lactococcus* sp. on the fact that he could get it to grow at 45 °C which, characteristically lactococci will not and growth in 6.5% NaCl which at that time, could not be accomplished with any of the known species of (maximum 4%) (Kusuda *et al.* 1991). Of the 13 isolates from the survey that were presumptively identified as belonging to the genus *Lactococcus*, none of which were identified as *Lactococcus garvieae*, 7 grew in 6.5% NaCl which none of the known species will do except *Lactococcus garvieae* and must therefore be as yet unknown species of lactococcus.

Table 4.1.10 summarises the results of seven authors testing the same organism *Enterococcus seriolicida* (ATCC 49156) obtained from the culture collection and highlights some of the discrepancies in their findings. Despite the fact that Kusuda *et al.* (1991) found the type strain *Enterococcus seriolicida* YT3 produced acid from sorbitol, most other authors testing the same strain found that this did not occur, suggesting that discrepancies may occur depending upon the method used for testing (Teixeira *et al.* 1996; Toranzo *et al.* 1994a). Toranzo *et al.* (1994a) found that the method (conventional versus multi-test) used gave different results for the fermentation of lactose and sorbitol, but did not state which method produced which result.

Salati *et al.* (1996) used routine or conventional methods, API 20S (BioMerieux Vitek) and the Vitek automated system (BioMerieux Vitek) to identify the strain of *Enterococcus* sp. causing disease in Adriatic sturgeon (*Acipenser naccarii*) and to compare it to *Enterococcus seriolicida* (ATCC 49156) as well as two other strains of *Enterococcus seriolicida* (E-092 and E-014) isolated from diseased yellowtail, in Japan, in 1992. Contrary to most findings, they found, in the text and the table, that the sturgeon pathogen, *Enterococcus seriolicida* (ATCC 49156) and one of the other

strains were VP negative although from the profile number given for the API 20S (5003511, 5043110 & 5043110 respectively) they would have been VP positive. Also, contrary to most findings they found all the three strains of *Enterococcus seriolicida,* (ATCC 49156, E-092 and E-014) to be PYR negative.

Table 4.1.11 summarises the characterisation results of the Australian isolates, causing disease in rainbow trout, compared to the results of organisms causing "streptococcosis" in the same or other fish species worldwide. It can been seen from these results that many of the reactions are similar to the findings of this study. Given the discrepancies found when different authors tested the same organism *Enterococcus seriolicida* (ATCC 49156), it is not unreasonable to suggest that some of these organisms causing streptococcosis worldwide may, in fact, be the same as the Australian isolate of *Lactococcus garvieae* .

## 4.2 Genotypic differentiation of selected isolates

### Introduction

Polymerase chain reaction (PCR) (Mullis, 1990) is a very powerful tool to enzymatically amplify a target nucleic acid sequence. It normally involves cyclic amplification of the target DNA in the presence of a pair of synthetic oligonucleotide primers complementary to sequences flanking the target DNA and *Taq* (*Thermus aquaticus*) DNA polymerase. The polymerase chain reaction allows the *in vitro* replication of defined DNA sequences, and so is of great value in bacterial classification and identification (Logan, 1994).

The random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR), which may also be termed arbitrarily primed polymerase chain reaction (AP-PCR) (Bej & Mahbubani, 1996), was employed to compare *Streptococcus sp.* biovar 1, associated with streptococcosis in rainbow trout, with environmental isolates and also with the type strains of related or possibly identical organisms. A PCR assay involves the use of temperature cycling to achieve primer-directed enzymatic amplification of desired segments of DNA.

The RAPD-PCR technique involves the use of single, short (usually 10-mer) random oligonucleotide primers and low stringency annealing conditions to amplify arbitrary DNA fragments from the genome of the organism (Figure 4.2.1). The primer anneals to the organism's template DNA, where a complementary sequence occurs, and, if two priming sites are sufficiently close, PCR will amplify the fragment between them to an extended copy of the target DNA. When the amplified fragments are separated by electrophoresis, they produce a readily distinguishable pattern of DNA bands which represent a DNA fingerprint that can be used to identify microbial taxa. A number of fragments or amplification products of various sizes may be produced, forming a RAPD 'fingerprint' that is specific for the particular DNA template used (Mileham, 1995; Bej & Mahbubani, 1996).

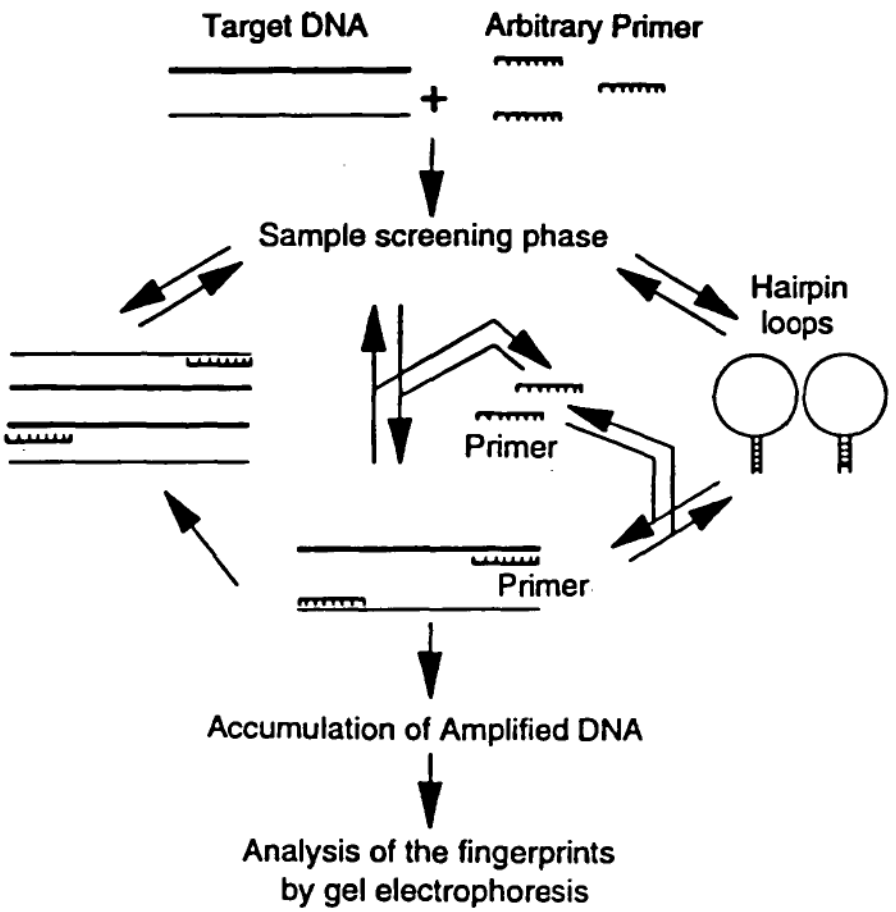



**Figure 4.2.1** Diagram of random amplified polymorphic DNA-PCR demonstrating the interactions between molecular species formed during DNA amplification with a single, arbitrary oligonucleotide primer. Following the template "screening" phase, a set of DNA fragments is synthesised. These first-round amplification products are initially single-stranded and have palindromic termini that allow formation of hairpin loops. In subsequent rounds of amplification, the products can be in the form of template-template and primer-template duplexes, as well as in single-strand and hairpin loops. The different species produced tend to establish an equilibrium while enzyme-anchoring and primer-extension transform the relatively rare primer-template duplexes into accumulating amplification products. (Bej & Mahbubani, 1996)

## Materials and Methods

### 4.2.1 Preparation of crude DNA

#### 4.2.1.1 Culture

The type strains of *Enterococcus seriolicida* (ATCC 49156) and *Lactococcus garvieae* (LMG 8893) were obtained from Mount Pleasant Laboratories and *Enterococcus seriolicida* YT3 was obtained from Japan. The ten fish-pathogenic strains of *Streptococcus* sp. biovar 1 obtained at the outset of this project, plus a further 18 isolates from diseased fish, animal faecal samples and environmental samples that were selected from isolates examined in Chapter 4.1 were included in this experiment. The identification codes, speciation and sources of these isolates are summarised in Table 4.2.1. The cultures in cryotubes were removed from long-term storage at -70°C and subcultured onto HBA (horse blood agar), incubated overnight at 35°C and examined for growth and purity. From these cultures the DNA for RAPD-PCR was to be extracted, so the growth purity check step was crucial.

#### 4.2.1.2 DNA extraction

The organisms were grown in 100mL of tryptone soya broth in 250mL Erhlenmeyer flasks at 35°C overnight with constant gentle agitation. The broth was aliquoted into two 50mL Greiner™ tubes and centrifuged at 5000g for 15min to pellet the cells. The pellet was resuspended in 2mL of lysis buffer (10mM Tris pH 9.5, 100mM EDTA, 1mg/mL lysozyme) and incubated on ice for 5min to loosen the cell walls. Sodium dodecyl sulphate (SDS) to a final concentration of 1% and proteinase K to a final concentration of 100µg/mL were added, and the sample placed into a 55°C waterbath until lysis was complete. It was necessary to further incubate overnight for complete clearing to occur. Proteins were precipitated by the addition of 0.33 volume of 6M NaOH. Lipids were removed by the addition of 1 volume chloroform, mixing for 15min and centrifugation at 4000g for 10min giving an interface of protein between the chloroform and aqueous DNA. Using a wide bore pipette, the aqueous DNA phase was removed to a sterile tube and incubated with RNase A (DNase free) at a concentration of 300µg/mL at room temperature for one hour. The resultant solution was transferred to pre-boiled lengths of dialysis tubing and dialysed against TE (Tris EDTA) buffer at 4°C overnight to reduce any residual EDTA that may interfere with the action of the *Taq* polymerase.

**Table 4.2.1 Enterococci and lactococci differentiated by RAPD-PCR**

| Code | Original Code | Original Species | Source |
|---|---|---|---|
| MP6 | V87/1039-1 | *Streptococcus* sp. biovar 1 | Mt. Pleasant Labs |
| MP9 | 88/3910-15 | *Streptococcus* sp. biovar 1 | Mt. Pleasant Labs |
| MP16 | 86/0290-1 | *Streptococcus* sp. biovar 1 | Mt. Pleasant Labs |
| MP17 | 89/1592-1 | *Streptococcus* sp. biovar 1 | Mt. Pleasant Labs |
| MP20 | 88/0756 | *Streptococcus* sp. biovar 1 | Mt. Pleasant Labs |
| MP21 | 88/3910-5 | *Streptococcus* sp. biovar 1 | Mt. Pleasant Labs |
| MP28 | 88/3625-1 | *Streptococcus* sp. biovar 1 | Mt. Pleasant Labs |
| MP36 | V83/35051-1 | *Streptococcus* sp. biovar 1 | Mt. Pleasant Labs |
| MP38 | 88/3425-6 | *Streptococcus* sp. biovar 1 | Mt. Pleasant Labs |
| MP39 | 88/3910-9 | *Streptococcus* sp. biovar 1 | Mt. Pleasant Labs |
| UT3B | YT-3 | *Enterococcus seriolicida* | Japan |
| UT6B | Pig | *Enterococcus. casseliflavus* | Winkleigh |
| UT8B | Pig | *Enterococcus faecalis* | Winkleigh |
| UT1C | Pig | *Lactococcus* sp. | Winkleigh |
| UT2C | Chicken | *Enterococcus faecium* | Winkleigh |
| UT4C | Mud | *Enterococcus* sp. | Cressy |
| UT1D | Cattle | *Lactococcus* sp. | Cressy |
| UT2D | Septic tank | *Lactococcus* sp. | Cressy |
| UT4D | Out flow | *Enterococcus* sp. | Cressy |
| UT8D | Out flow | *Enterococcus faecalis* | Bridport |
| UT9D | In flow | *Enterococcus* sp. | Bridport |
| UT4E | Pelican | *Lactococcus* sp. | Bridport |
| UT7E | Grow out tank | *Lactococcus* sp. | Bridport |
| UT9E | Sheep | *Lactococcus* sp. | Bridport |
| UT8F | Septic tank | *Lactococcus* sp. | Bridport |
| UT9F | Platypus | *Lactococcus* sp. | Deloraine |
| UT2G | Rainbow trout | *Enterococcus* sp. | Bridport |
| UT9H | In flow | unknown | Bridport |
| UT1I | Grow out tank | *Streptococcus* sp. | Bridport |
| UT2I | ATCC 49156 | *Enterococcus seriolicida* | Mt. Pleasant Labs |
| MP/LG | LMG 8893 | *Lactococcus garvieae* | Mt. Pleasant Labs |

The successful extraction of DNA was proven by loading a 1% agarose gel with 1μL and 5μL samples of crude DNA and *Hind*III λ made up to 12μL with 2μL of loading dye and the balance of sterile distilled water. The gel was run in TBE at 120 volts for approximately 1.5h and then stained with 2ng/mL ethidium bromide for 15min, destained in distilled water for 15min with gentle shaking and visualised and photographed using a transilluminator and camera.

*Hind*III λ ladder contains 23130, 9416, 6557, 4361, 2322, 2057, & 564 base pairs (bp). Assuming that there was a 1:1 ratio of each fragment, the loading of 500ng into each well (5μL) would give band DNA concentrations of 500 x 23130/48377ng etc. for all bands, that is a concentration of 23130bp would be 239ng . Therefore, by loading 1μL of marker ladder the top band concentration would be 48ng. Concentrations of DNA in the resultant extracts were estimated by visually comparing the intensity of the test bands with the intensity of the top band of the marker. The DNA was then diluted to give a final concentration of 5ng/μL in TE buffer. For example: for a band with equal intensity to the top band of the marker (50ng /μL ) the sample would be diluted 1:10. The sample was then stored at -20°C.

### 4.2.2 Optimisation of the reagents for RAPD-PCR

A specific mixture of reagents was required for the PCR protocol. This includes the extracted DNA, magnesium chloride and *Taq* DNA polymerase, all of which need to be optimised before the assay could be run, primers, dNTPs and reaction buffer. When the optimum concentrations for the reaction mixture had been determined, the protocol for PCR (section 4.2.3) was performed. Oligonucleotide primers (OPB-01 to OPB-20) were obtained in the form of a kit from Operon Technologies.

#### 4.2.2.1 Magnesium Chloride

Magnesium chloride is required in the reaction as a co-factor for *Taq* DNA polymerase. It also stabilises the primer/template complex. In order to identify the optimum quantity of $MgCl_2$ required for the assay, 6 duplicate reactions were set up using 2 randomly selected DNA samples, and volumes of $MgCl_2$ of 0, 2, 4, 6, 8, 10, and 12μL (equivalent to 0, 1.25, 2.5, 3.75, 5, 6.25, and 7.5 mM) per 40μL, reaction.

The reaction mixture used for the optimisation contained 4µL of 10X reaction buffer (final concentrations of 67 mM Tris {pH 8.8}, 16.6 mM ammonium sulphate, 0.45% Triton X-100 and 20 µg $l^{-1}$ gelatin), 4µL (250 µM) dNTP of dNTP mix (containing 10µL each of 4 deoxynucleoside 5'-triphosphates, dATP, dCTP, dGTP & dTTP plus 360µL $dDH_2O$), 1µL (50 pmol) of primer (primer OPB7 was randomly selected to use for the optimisation), 7µl of crude DNA (samples UT9D and UT2G were randomly selected for use in the optimisation), 2.2u *Taq* polymerase (2µL) and made up to 40µL with $dDH_2O$. The protocol for PCR (section 4.3) was then followed.

#### 4.2.2.2 *Taq* DNA Polymerase

*Taq* DNA polymerase is a thermostable enzyme prepared from the thermophilic ("heat-loving") bacterium *Thermus aquaticus* which replicates DNA at 74°C. The ability of the enzyme to survive multiple rounds of temperature cycling makes it extremely useful in PCR and temperature cycling. The definition of a unit of *Taq* DNA polymerase is the amount of enzyme required to catalyse the incorporation of 10 nmol of dNTP into acid-insoluble form in 30min at 74°C in the presence of the reaction buffer. For the optimisation of the *Taq,* 5 duplicate amounts of *Taq* of 0, 1.0, 1.5, 2.0 and 2.5 units were used. The optimum amount of magnesium chloride (as determined in an earlier experiment - section 4.2.2.1) was 6µL and the rest of the reagents other than the *Taq* were the same as for the $MgCl_2$ optimisation. *Taq* (concentration 5.5units/µL) was diluted 1:10 with $dDH_2O$ so that 2µL contained ≈1 unit. The reaction containing no *Taq* was made up to 40µL with $dDH_2O$ and the other *Taq* concentrations 2, 3, 4, and 5µL of *Taq* were added with the balance $dDH_2O$ to make 40µL. The protocol for PCR (section 4.2.3) was then followed.

### 4.2.3 Extracted DNA

The extracted DNA samples containing 5ng/µL of DNA were the final parameter for optimisation. The reactions were set up to contain the optimal amount of $MgCl_2$ (6µl) and Taq DNA polymerase (4µL) as determined in earlier experiments (see sections 4.2.1& 4.2.2).and the rest of the reagents other than the extracted DNA were the same as for the $MgCl_2$ and *Taq* polymerase DNA optimisations (4µL of reaction buffer, 4µL of dNTP and 1µL of primer) . Samples were prepared to contain amounts

between 0ng to 50ng of extracted 5ng/μL DNA in the reaction medium (0μL being a negative control) with the balance $dDH_2O$ to bring the volume to 40μL (Table 4.2.2 ). The protocol for PCR (section 4.2.3) was then followed.

**Table 4.2.2 Summary of the concentrations and volumes used in the DNA optimisation**

| Amount of DNA in ηg | Volume of DNA in μL | Volume of $dDH_2O$ in μL |
|---|---|---|
| 0 | 0 | 21 |
| 10 | 2 | 19 |
| 20 | 4 | 17 |
| 30 | 6 | 15 |
| 35 | 7 | 14 |
| 40 | 8 | 13 |
| 50 | 10 | 11 |

### 4.2.3 PCR protocol

The reaction mixtures were prepared in 0.5mL eppendorf tubes (Trace Plastics ™) and cycled in a Hybaid Omnigene™ temperature cycler for 45 cycles of 94°C for 1min for denaturing of DNA, 36°C for 1min for primer annealing and 72°C for 2min for polymerisation (extension) (Figure 4.2.2). This total length of this process took approximately 8 to 12h to complete depending upon the ambient temperature at the time.

The PCR amplification products were prepared for electrophoresis by mixing 10μL of the product with 2μL of bromophenol blue loading dye in a microtitre plate. *Hae*III cut ØX 174 (Promega™) was used as a DNA size marker. A blank sample of the reaction solution was run in the well between the marker well and the first test well to monitor for any contaminating DNA in the reaction solution. A 1.4% agarose gel was prepared by dissolving 2.8g of agarose in 200mL of 1X TBE (Tris, boric acid, EDTA) buffer by heating to boiling in a microwave oven, cooling to approximately 50°C and pouring into a prepared 20x20cm gel tray, placing 2x22-well combs in position and allowing to set for 30min. When set the combs and the tape used to contain the gel were removed and the gel placed into a submarine electrophoresis bath of TBE (Tris,

boric acid & EDTA) buffer making sure that the surface of the gel was covered with a thin layer of the buffer.

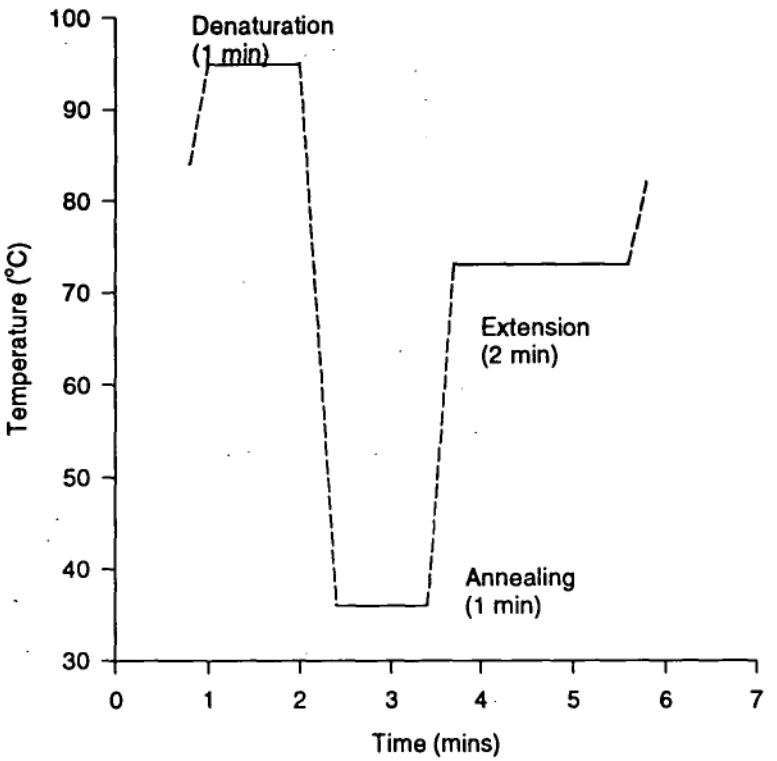


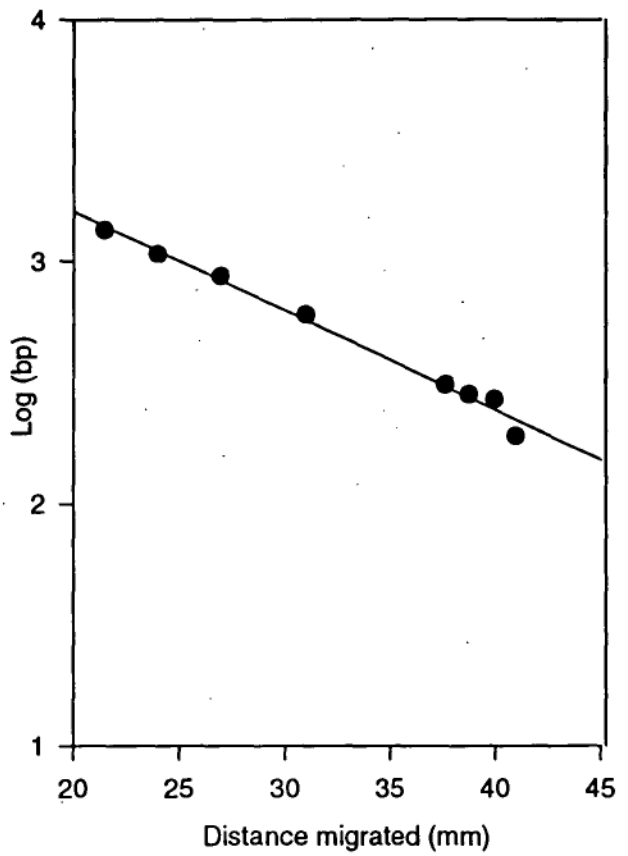

**Figure 4.2.2** Diagram of the PCR cycling process

The wells were then loaded with 12μL of dyed PCR amplification product and electrophoresed at 120v for approximately 2.5h. The gel was then stained with 2ug/mL ethidium bromide for 15min with gentle shaking, washed for 15min in distilled water with gentle agitation, visualised over UV light on a transilluminator and photographed.

### 4.2.4 Analysis of the gel

Estimation of the size (molecular weight) of the RAPD bands or fingerprints was performed by comparing the known size of the marker bands (*Hae*III cut ØX 174) to the size of the test bands by comparing the location (i.e. distance travelled) of the bands on the gel. This was performed by drawing a line along the front of the

inoculation wells on every photograph and measuring the distance migrated by the band from the line to the obverse side of the band using vernier callipers. The log (molecular weight in kilobases) was plotted against the distance the DNA fragment moved in mm (Figure 4.2.3).




| Size (bp) | Log (bp) |
|---|---|
| 1353 | 3.13 |
| 1078 | 3.03 |
| 872 | 2.94 |
| 603 | 2.78 |
| 310 | 2.49 |
| 281 | 2.45 |
| 271 | 2.43 |
| 194 | 2.28 |
| 112 | 2.05 |
| 72 | 1.86 |

**Figure 4.2.3** Typical example of the graphs used to calculate the relative size of the bands

The regression line of the plot was then used to calculate the size of the other base pairs using the regression equation:

$$y = mx + c$$

where y is log of the base pair, m is the slope, x is the distance migrated in mm and c

is the intercept on the y axis. This was repeated for all 22 photographs and the $r^2$ for all of the photographs was consistently greater than 0.98.

## Results

### 4.2.1 Preparation of crude DNA

#### 4.2.1.1 Culture

Cultures were examined and found to be pure, which is an important step because any contamination would result in amplification of foreign DNA.

#### 4.2.1.2 DNA extraction

Photographs taken of the gels were examined for the presence of bands of extracted crude DNA (Figure 4.2.4). These showed that DNA was successfully extracted from the bacteria in lanes 5-12, 14 & 16-22 but not from the bacteria in the other lanes. The extraction process was repeated on those bacteria and was mostly successful the second time. Lanes 1 & 2 contained the DNA marker. Comparing the intensity of the bands to that of the markers indicated that several of the extracts needed to be diluted to the desired concentration of 5ng in TE buffer (see Methods & Material 4.2.3).

### 4.2.2 Optimisation of the reagents for RAPD-PCR

#### 4.2.2.1 Magnesium chloride

As can be seen in Figure 4.2.5, the optimisation experiment showed that, for the DNA extracted from sample UT9D, the best resolution was obtained with 6µL (3.75 mM) of $MgCl_2$, because the bands were clearly visible and showed good replication. Sample UT9D and a volume of 6µL of $MgCl_2$ was used for the remainder of the optimisation experiments.

#### 4.2.2.2 *Taq* DNA Polymerase

Figure 4.2.6 shows that 2 units (4µL) of *Taq* DNA polymerase gave the clearest banding, therefore this concentration of *Taq* was selected as optimum concentration for the assay.

#### 4.2.2.3 Extracted DNA

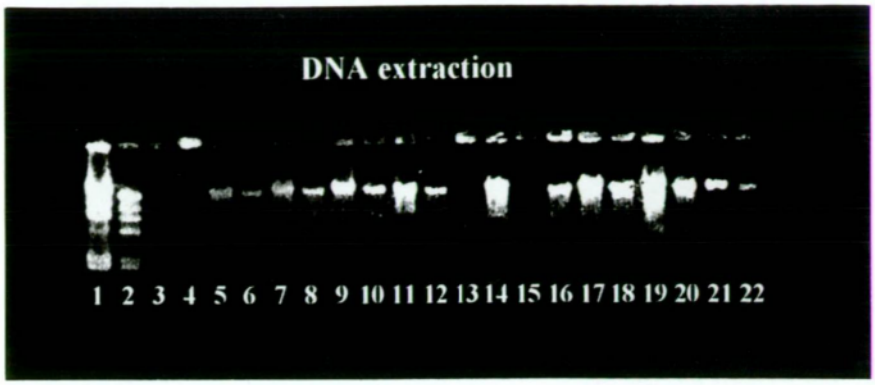
Figure 4.2.7 shows that all the concentrations of DNA gave clear banding and duplication so it was decided to run the RAPD assay with 35ng (7μL) as the optimum concentration. Also, note that the reaction containing no extracted DNA gave some banding, indicating that the reaction was contaminated with DNA. This was overcome by discarding all reagents and restarting with fresh solutions.

### 4.2.3 PCR protocol

Results of the amplification of the crude DNA varied between the 20 different primers, which was as to be expected (Table 4.2.3). Eight of the primers failed to amplify any products while six of the primers only showed some indistinct amplification products. These primers were not tested further. The remaining six primers OPBs 7, 9, 10, 12, 17, & 18 showed promising discriminatory banding patterns between the *Streptococcus* sp. biovar 1 and the reference type strains. These primers were then repeat tested (recycled or replicated) to establish if the banding was reproducible. It was found that, although the entire fingerprint was not always reproducible with all of the Australian isolates of *Streptococcus* sp. biovar 1, *Enterococcus seriolicida* (ATCC 49156) and *Lactococcus garvieae* (LMG 8893), some of the bands were distinctly evident upon repeat testing. To confirm that the bands were the same size or molecular weight, it was necessary to analyse the gel images graphically.

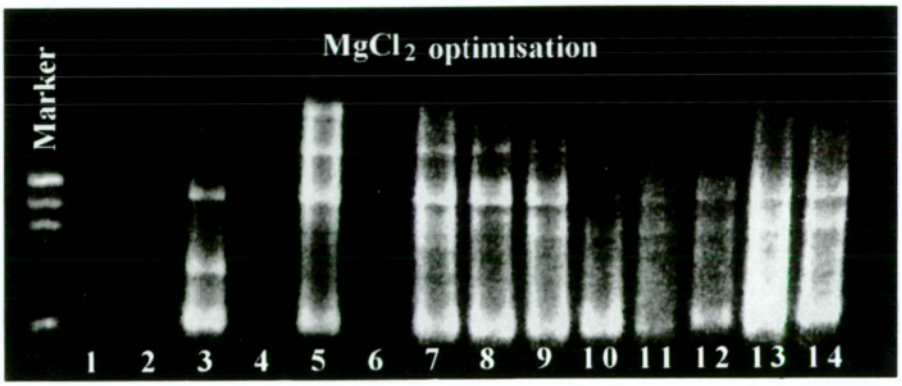
### 4.2.4 Analysis of the gel

A separate plot for each gel photograph was necessary because of the inconsistency between photos, mainly due to variation in the positioning of the camera. The results of the reproducible bands found with each primer are shown in Table 4.2.3. Primers 07, 09, 10 and 12 showed consistent banding, with the most of the *Streptococcus* sp. biovar 1 isolates having the same pattern or fingerprint as *Enterococcus seriolicida* (ATCC 49156) (UT3B and UT2I) and *Lactococcus garvieae* (LMG 8893). Primer OPB 17 had two major fragments at ≈ 655 and 275bp with the same fingerprint for most of the type strains, but also gave some good reproducible bands with a few of the *Streptococcus* sp. biovar 1 isolates and for *Enterococcus seriolicida* (ATCC



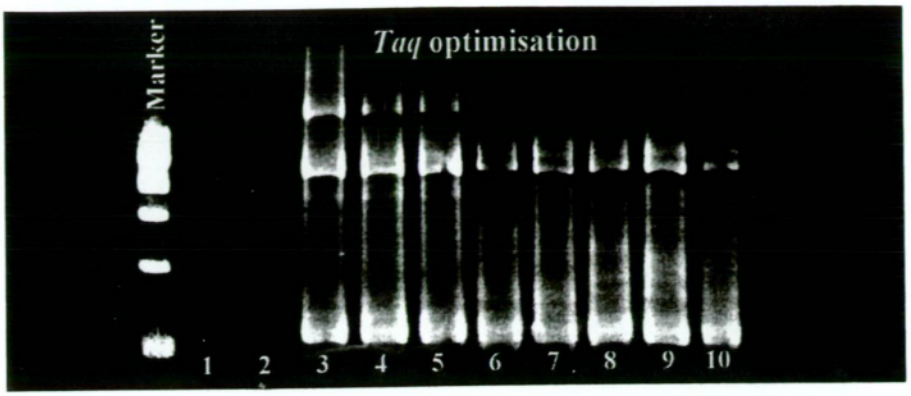

| Lane | Sample | Lane | Sample | Lane | Sample |
|---|---|---|---|---|---|
| 1 | *Hin*III λ 500n | 9 | MPL17 5 µL | 17 | MPL36 5 µL |
| 2 | *Hin*III λ 100n | 10 | MPL17 1 µL | 18 | MPL36 1 µL |
| 3 | MPL6 5 µL | 11 | MPL20 5 µL | 19 | MPL38 5 µL |
| 4 | MPL6 1 µL | 12 | MPL20 1 µL | 20 | MPL38 1 µL |
| 5 | MPL9 5 µL | 13 | MPL21 5 µL | 21 | MPL39 5 µL |
| 6 | MPL9 1 µL | 14 | MPL21 1 µL | 22 | MPL39 1 µL |
| 7 | MPL16 5 µL | 15 | MPL28 5 µL | | |
| 8 | MPL16 1 µL | 16 | MPL28 1 µL | | |

**Figure 4.2.4** Gel showing the successful extraction of DNA from the bacterial cultures in lanes 5-12, 14, & 16-22



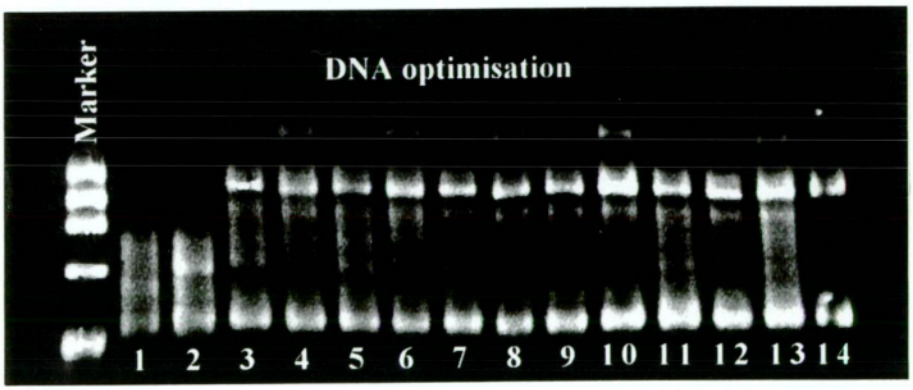

| Lane | $MgCL_2$ in µL | Lane | $MgCL_2$ in µL | Lane | $MgCL_2$ in µL |
|---|---|---|---|---|---|
| 1 | 0 | 6 | 4 | 11 | 10 |
| 2 | 0 | 7 | 6 | 12 | 10 |
| 3 | 2 | 8 | 6 | 13 | 12 |
| 4 | 2 | 9 | 8 | 14 | 12 |
| 5 | 4 | 10 | 8 | 15 | |

**Figure 4.2.5** Gel showing the optimisation of $MgCl_2$ concentration to be used in the random amplified polymorphic DNA protocol with UT9D



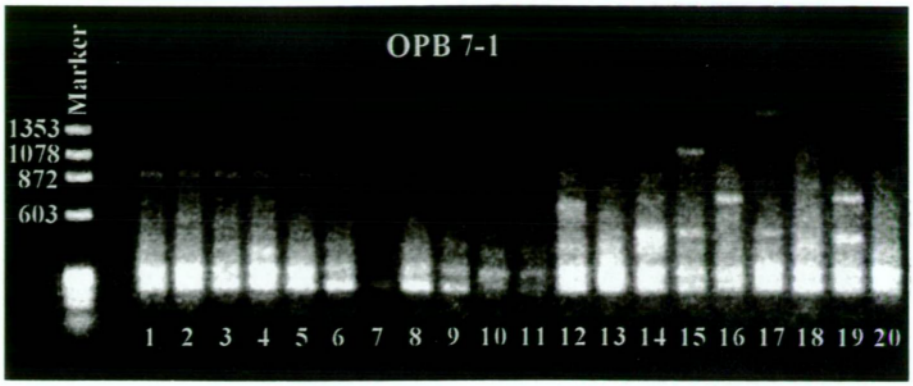

| Lane | Units of *Taq* | Lane | Units of *Taq* |
|---|---|---|---|
| 1 | 0 | 6 | 1.5 |
| 2 | 0 | 7 | 2.0 |
| 3 | 1.0 | 8 | 2.0 |
| 4 | 1.0 | 9 | 2.5 |
| 5 | 1.5 | 10 | 2.5 |

**Figure 4.2.6** Gel showing the optimisation of concentration of *Taq* DNA polymerase to be used in the random amplified polymorphic DNA protocol with UT9D



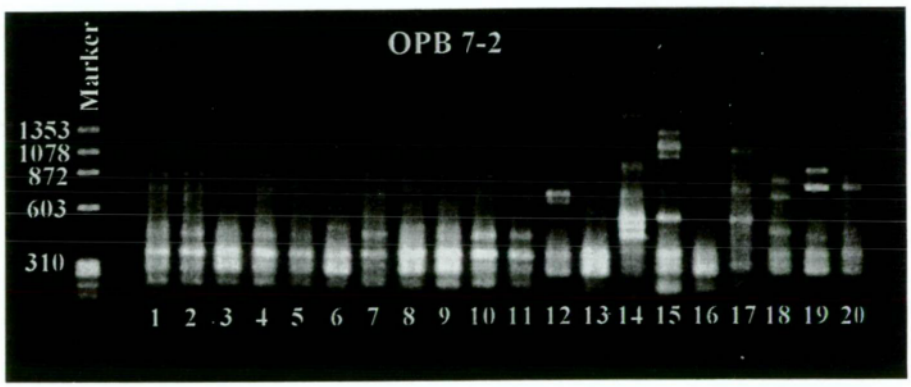

| Lane | DNA in ng | Lane | DNA in ng | Lane | DNA in ng |
|---|---|---|---|---|---|
| 1 | 0 | 6 | 20 | 11 | 40 |
| 2 | 0 | 7 | 30 | 12 | 40 |
| 3 | 10 | 8 | 30 | 13 | 50 |
| 4 | 10 | 9 | 35 | 14 | 50 |
| 5 | 20 | 10 | 35 | 15 | |

**Figure 4.2.7** Gel showing the optimisation of the concentration of DNA to be used in the random amplified polymorphic DNA protocol with UT9D



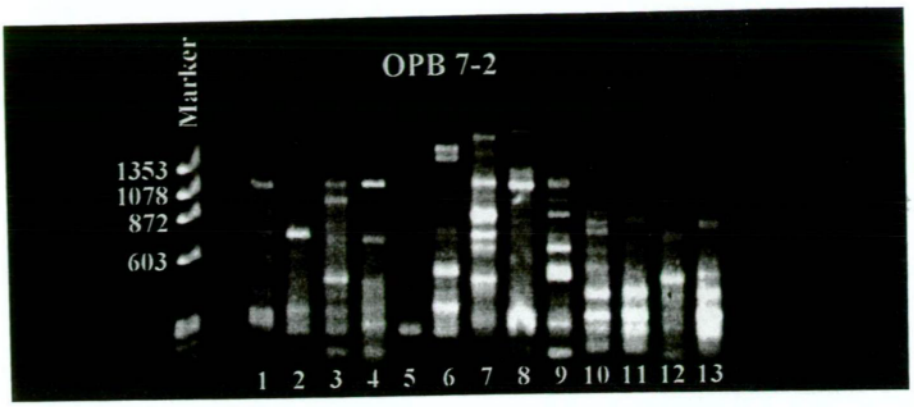

| Lane | Sample | Lane | Sample | Lane | Sample | Lane | Sample |
|---|---|---|---|---|---|---|---|
| 1 | MP6 | 6 | MP21 | 11 | UT3B | 16 | UT4C |
| 2 | MP9 | 7 | MP28 | 12 | UT6B | 17 | UT7E |
| 3 | MP16 | 8 | MP36 | 13 | UT8B | 18 | UT9F |
| 4 | MP17 | 9 | MP38 | 14 | UT1C | 19 | UT2G |
| 5 | MP20 | 10 | MP39 | 15 | UT2C | 20 | UT2I |

**Figure 4.2.8** Samples tested with primer OPB 7 (1st cycle)




| Lane | Sample | Lane | Sample | Lane | Sample | Lane | Sample |
|---|---|---|---|---|---|---|---|
| 1 | MP6 | 6 | MP21 | 11 | UT3B | 16 | UT4C |
| 2 | MP9 | 7 | MP28 | 12 | UT6B | 17 | UT1D |
| 3 | MP16 | 8 | MP36 | 13 | UT8B | 18 | UT2D |
| 4 | MP17 | 9 | MP38 | 14 | UT1C | 19 | UT4D |
| 5 | MP20 | 10 | MP39 | 15 | UT2C | 20 | UT8D |

**Figure 4.2.9** Samples tested with primer OPB 7 (2nd cycle)A




| Lane | Sample | Lane | Sample | Lane | Sample |
|---|---|---|---|---|---|
| 1 | UT9D | 6 | UT9F | 11 | MP28 |
| 2 | UT4E | 7 | UT2G | 12 | UT7E |
| 3 | UT7E | 8 | UT9H | 13 | MP/LG |
| 4 | UT9E | 9 | UT1I | | |
| 5 | UT8F | 10 | UT2I | | |

**Figure 4.2.10** Samples tested with primer OPB 7 (2nd cycle)B

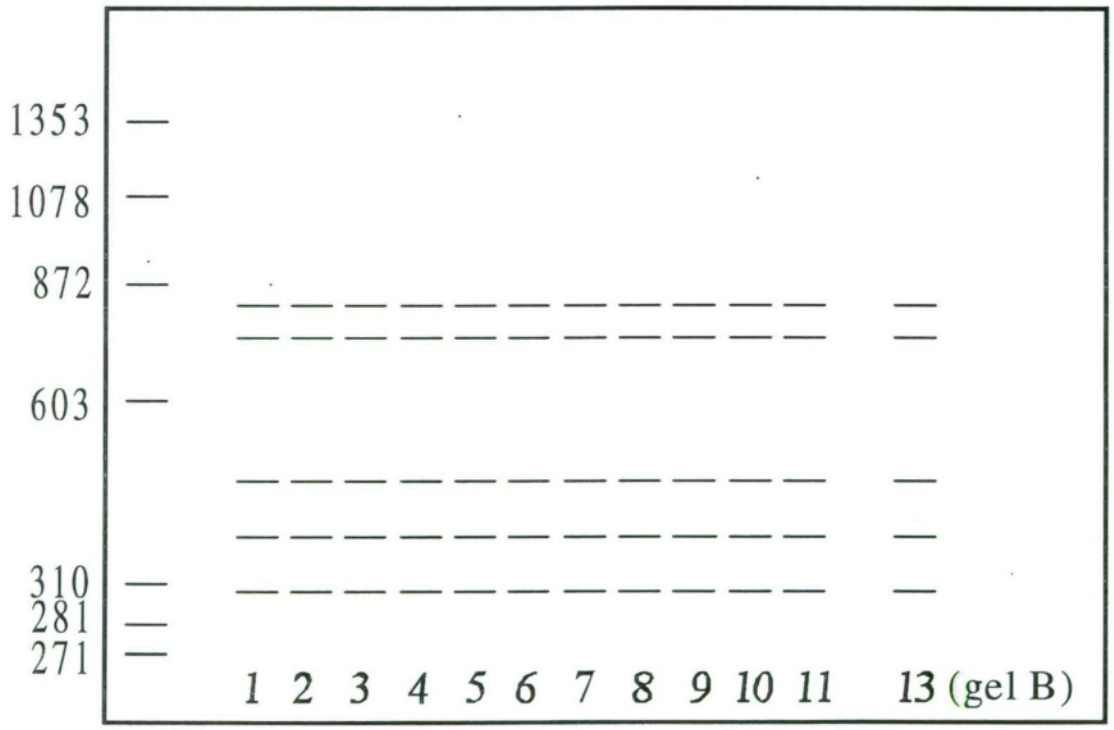
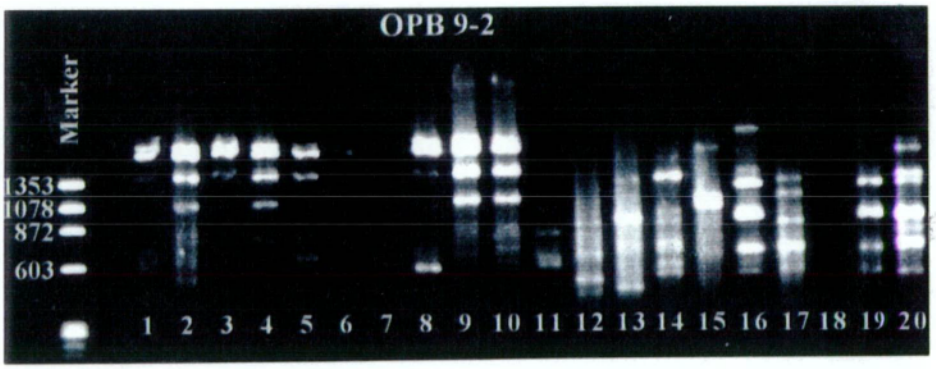



**Figure 4.2.11** Diagrammatic representation of the reproducible bands with primer OPB 7 (2nd Cycle), showing the *Streptococcus* sp. biovar 1 strains (lanes 1 to 10) *Enterococcus seriolicida* (ATCC 49156) (lane 11) and *Lactococcus garvieae* (LMG 8893) (lane 13, gel B)

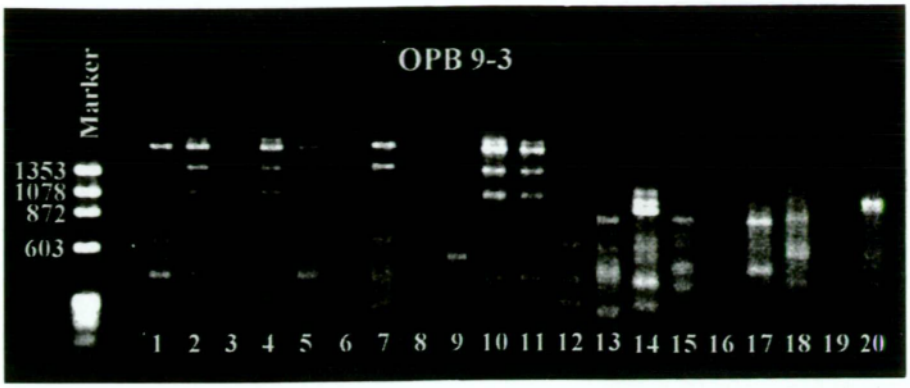

| Lane | Sample | Lane | Sample | Lane | Sample | Lane | Sample |
|---|---|---|---|---|---|---|---|
| 1 | MP6 | 6 | MP21 | 11 | UT3B | 16 | UT4E |
| 2 | MP9 | 7 | MP28 | 12 | UT6B | 17 | UT7E |
| 3 | MP16 | 8 | MP36 | 13 | UT8B | 18 | UT9F |
| 4 | MP17 | 9 | MP38 | 14 | UT1C | 19 | UT2G |
| 5 | MP20 | 10 | MP39 | 15 | UT2C | 20 | UT2I |

**Figure 4.2.12** Samples tested with primer OPB 9 (1st cycle)



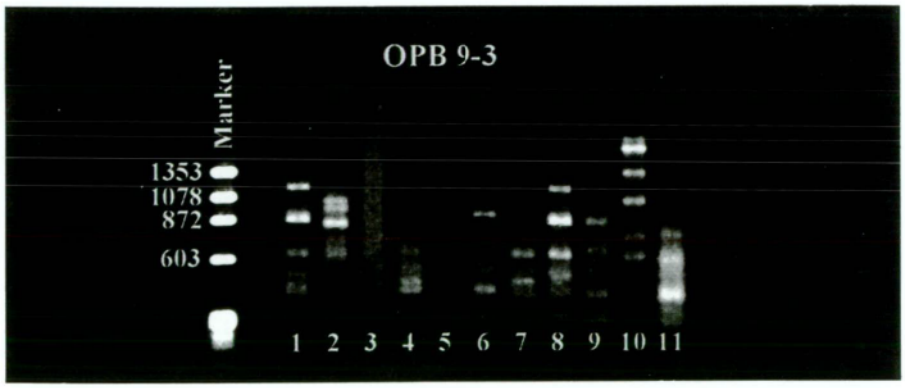

| Lane | Sample | Lane | Sample | Lane | Sample | Lane | Sample |
|---|---|---|---|---|---|---|---|
| 1 | MP6 | 6 | MP21 | 11 | UT4C | 16 | UT9D |
| 2 | MP9 | 7 | MP36 | 12 | UT1D | 17 | UT9E |
| 3 | MP16 | 8 | MP38 | 13 | UT2D | 18 | UT8F |
| 4 | MP17 | 9 | MP39 | 14 | UT4D | 19 | UT9H |
| 5 | MP20 | 10 | UT2I | 15 | UT8D | 20 | UT1I |

**Figure 4.2.13** Samples tested with primer OPB 9 (2nd cycle)




| Lane | Sample | Lane | Sample | Lane | Sample | Lane | Sample |
|---|---|---|---|---|---|---|---|
| 1 | MP6 | 6 | MP21 | 11 | UT3B | 16 | UT4C |
| 2 | MP9 | 7 | MP28 | 12 | UT6B | 17 | UT1D |
| 3 | MP16 | 8 | MP36 | 13 | UT8B | 18 | UT2D |
| 4 | MP17 | 9 | MP38 | 14 | UT1C | 19 | UT4D |
| 5 | MP20 | 10 | MP39 | 15 | UT2C | 20 | UT8D |

**Figure 4.2.14** Samples tested with primer OPB 9 (3rd cycle)




| Lane | Sample | Lane | Sample | Lane | Sample |
|---|---|---|---|---|---|
| 1 | UT9D | 6 | UT9F | 11 | MP/LG |
| 2 | UT4E | 7 | UT2G | | |
| 3 | UT7E | 8 | UT9H | | |
| 4 | UT9E | 9 | UT1I | | |
| 5 | UT8F | 10 | UT2I | | |

**Figure 4.2.15** Samples tested with primer OPB 9 (3rd cycle)

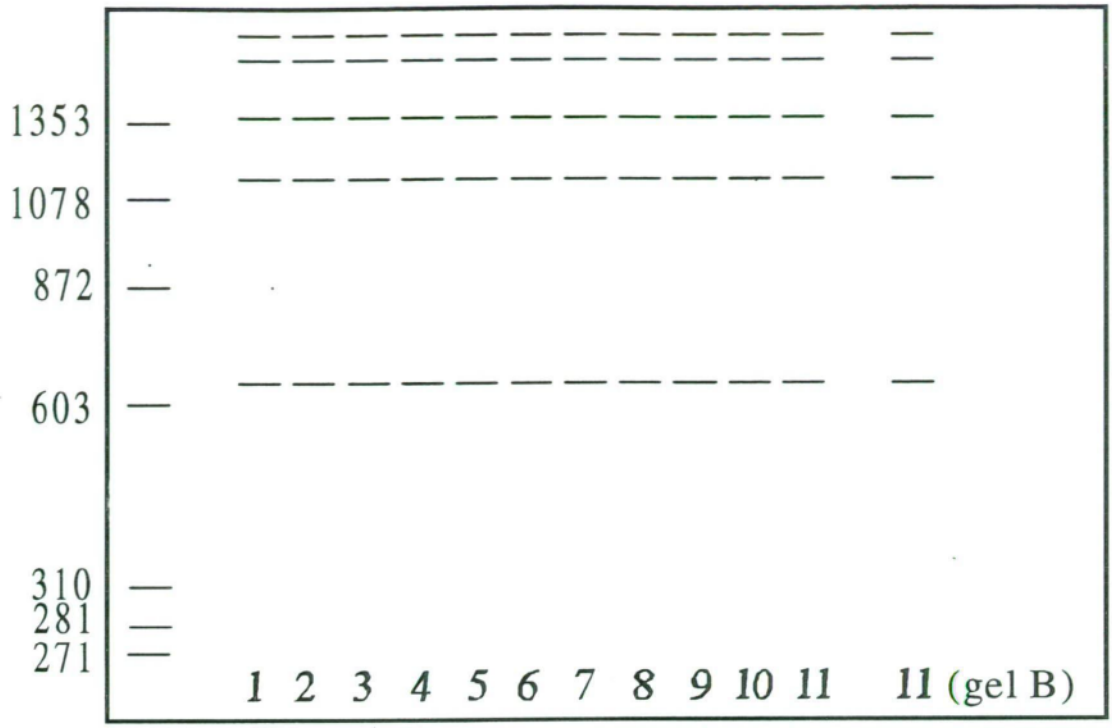
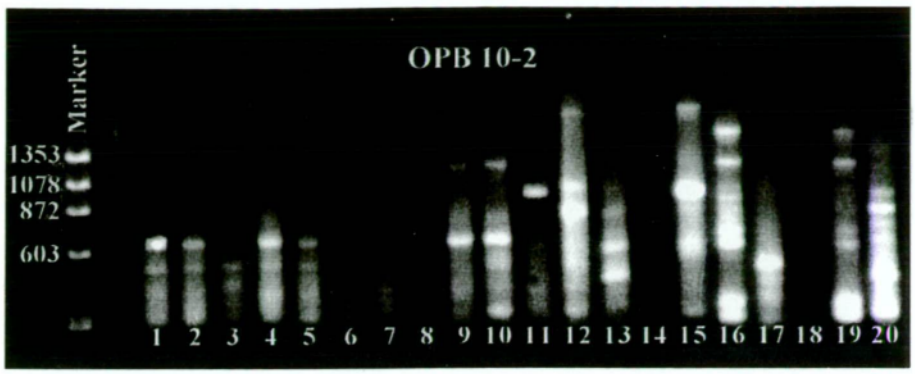



**Figure 4.2.16** Diagrammatic representation of the reproducible bands with primer OPB 9 (3rd Cycle), showing the *Streptococcus* sp. biovar 1 strains (lanes 1 to 10) *Enterococcus seriolicida* (ATCC 49156) (lane 11) and *Lactococcus garvieae* (LMG 8893) (lane 11, gel B)


| Lane | Sample | Lane | Sample | Lane | Sample | Lane | Sample |
|---|---|---|---|---|---|---|---|
| 1 | MP6 | 6 | MP21 | 11 | UT3B | 16 | UT4E |
| 2 | MP9 | 7 | MP28 | 12 | UT6B | 17 | UT7E |
| 3 | MP16 | 8 | MP36 | 13 | UT8B | 18 | UT9F |
| 4 | MP17 | 9 | MP38 | 14 | UT1C | 19 | UT2G |
| 5 | MP20 | 10 | MP39 | 15 | UT2C | 20 | UT2I |

**Figure 4.2.17** Samples tested with primer OPB10 (1st cycle)

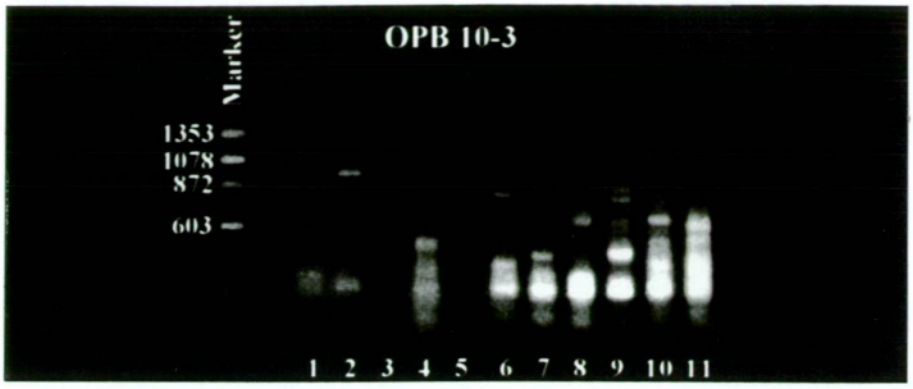



| Lane | Sample | Lane | Sample | Lane | Sample | Lane | Sample |
|---|---|---|---|---|---|---|---|
| 1 | MP6 | 6 | MP21 | 11 | UT4C | 16 | UT9D |
| 2 | MP9 | 7 | MP36 | 12 | UT1D | 17 | UT9E |
| 3 | MP16 | 8 | MP38 | 13 | UT2D | 18 | UT8F |
| 4 | MP17 | 9 | MP39 | 14 | UT4D | 19 | UT9H |
| 5 | MP20 | 10 | UT2I | 15 | UT8D | 20 | UT1I |

**Figure 4.2.18** Samples tested with primer OPB 10 (2nd cycle)


| Lane | Sample | Lane | Sample | Lane | Sample | Lane | Sample |
|---|---|---|---|---|---|---|---|
| 1 | MP6 | 6 | MP21 | 11 | UT3B | 16 | UT4C |
| 2 | MP9 | 7 | MP28 | 12 | UT6B | 17 | UT1D |
| 3 | MP16 | 8 | MP36 | 13 | UT8B | 18 | UT2D |
| 4 | MP17 | 9 | MP38 | 14 | UT1C | 19 | UT4D |
| 5 | MP20 | 10 | MP39 | 15 | UT2C | 20 | UT8D |

**Figure 4.2.19** Samples tested with primer OPB 10 (3rd cycle)A




| Lane | Sample | Lane | Sample | Lane | Sample |
|---|---|---|---|---|---|
| 1 | UT9D | 6 | UT9F | 11 | MP/LG |
| 2 | UT4E | 7 | UT2G | | |
| 3 | UT7E | 8 | UT9H | | |
| 4 | UT9E | 9 | UT1I | | |
| 5 | UT8F | 10 | UT2I | | |

**Figure 4.2.20** Samples tested with primer OPB10 (3rd cycle)B

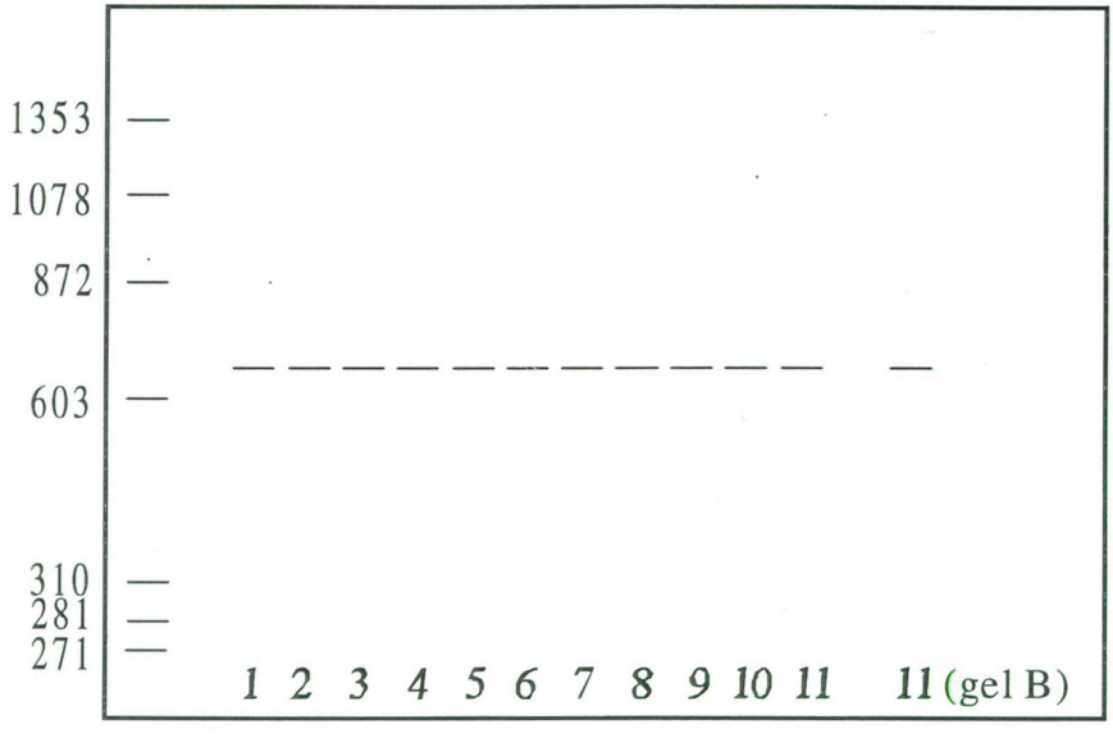
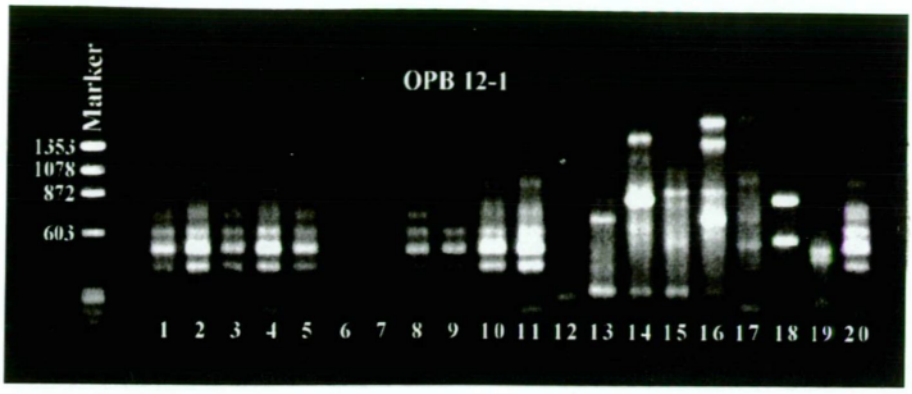
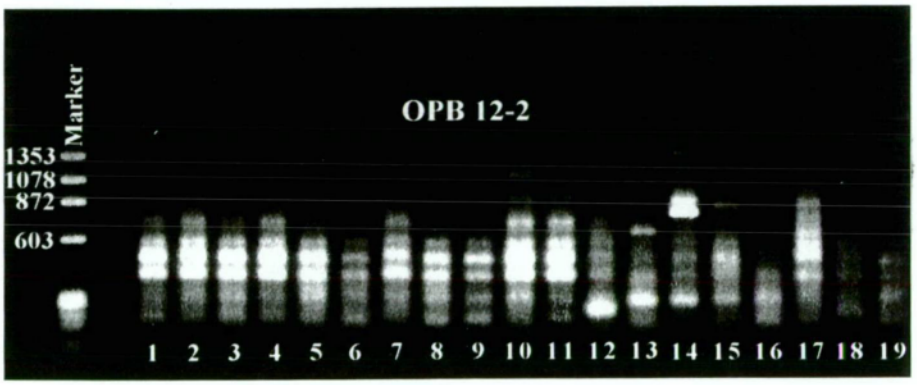



**Figure 4.2.21** Diagrammatic representation of the reproducible bands with primer OPB 10 (3rd Cycle), showing the *Streptococcus* sp. biovar 1 strains (lanes 1 to 10) *Enterococcus seriolicida* (ATCC 49156) (lane 11) and *Lactococcus garvieae* (LMG 8893) (lane 11, gel B)



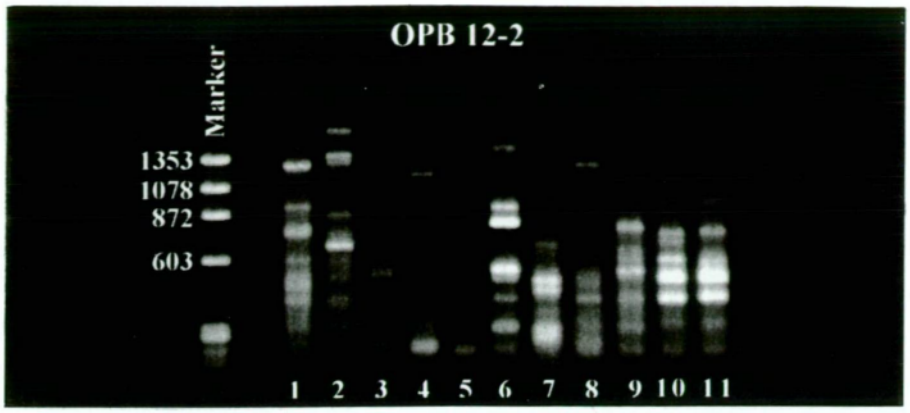

| Lane | Sample | Lane | Sample | Lane | Sample | Lane | Sample |
|---|---|---|---|---|---|---|---|
| 1 | MP6 | 6 | MP21 | 11 | UT3B | 16 | UT4C |
| 2 | MP9 | 7 | MP28 | 12 | UT6B | 17 | UT7E |
| 3 | MP16 | 8 | MP36 | 13 | UT8B | 18 | UT9F |
| 4 | MP17 | 9 | MP38 | 14 | UT1C | 19 | UT2G |
| 5 | MP20 | 10 | MP39 | 15 | UT2C | 20 | UT2I |

**Figure 4.2.22** Samples tested with primer OPB 12 (1st cycle)




| Lane | Sample | Lane | Sample | Lane | Sample | Lane | Sample |
|---|---|---|---|---|---|---|---|
| 1 | MP6 | 6 | MP21 | 11 | UT3B | 16 | UT4C |
| 2 | MP9 | 7 | MP28 | 12 | UT6B | 17 | UT1D |
| 3 | MP16 | 8 | MP36 | 13 | UT8B | 18 | UT2D |
| 4 | MP17 | 9 | MP38 | 14 | UT1C | 19 | UT4D |
| 5 | MP20 | 10 | MP39 | 15 | UT2C | 20 | UT8D |

**Figure 4.2.23** Samples tested with primer OPB 12 (2nd cycle)A




| Lane | Sample | Lane | Sample | Lane | Sample |
|---|---|---|---|---|---|
| 1 | UT9D | 6 | UT9F | 11 | MP/LG |
| 2 | UT4E | 7 | UT2G | | |
| 3 | UT7E | 8 | UT9H | | |
| 4 | UT9E | 9 | UT1I | | |
| 5 | UT8F | 10 | UT2I | | |

**Figure 4.2.24** Samples tested with primer OPB12 (2nd cycle)B

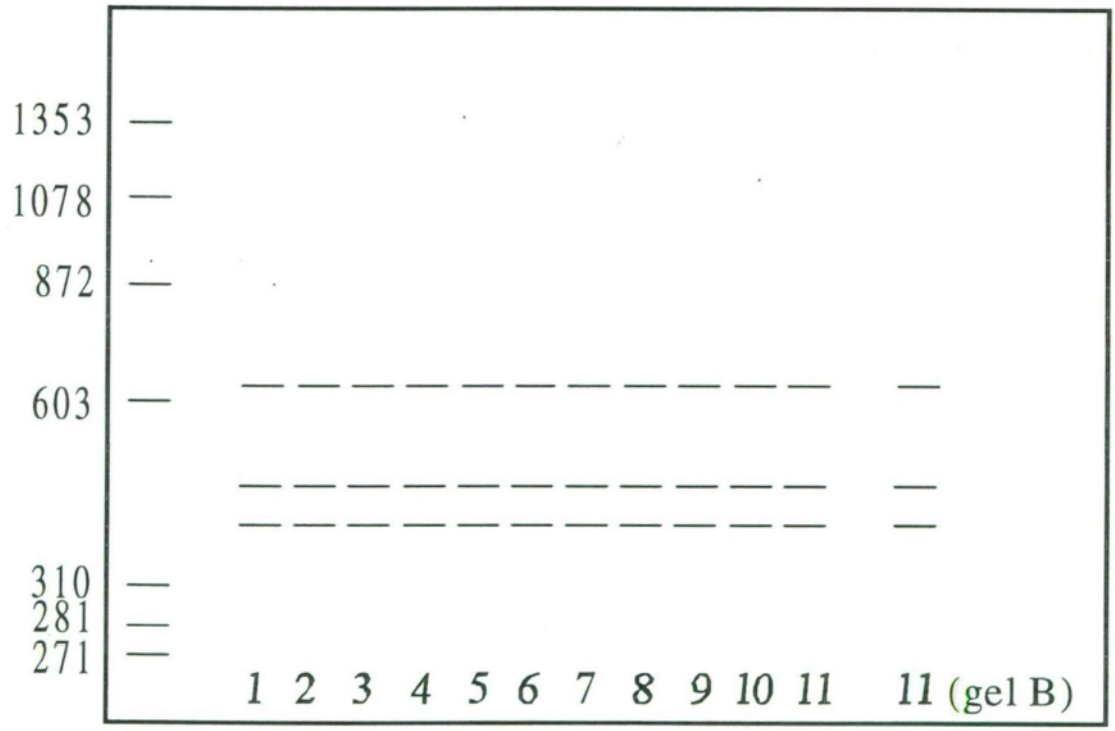
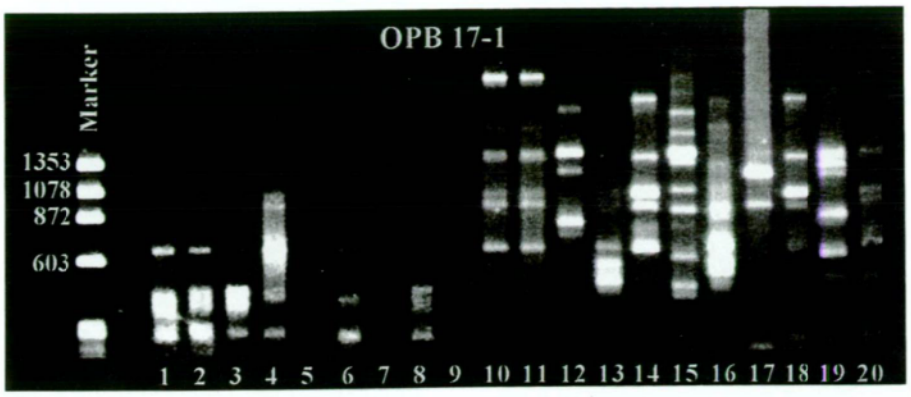
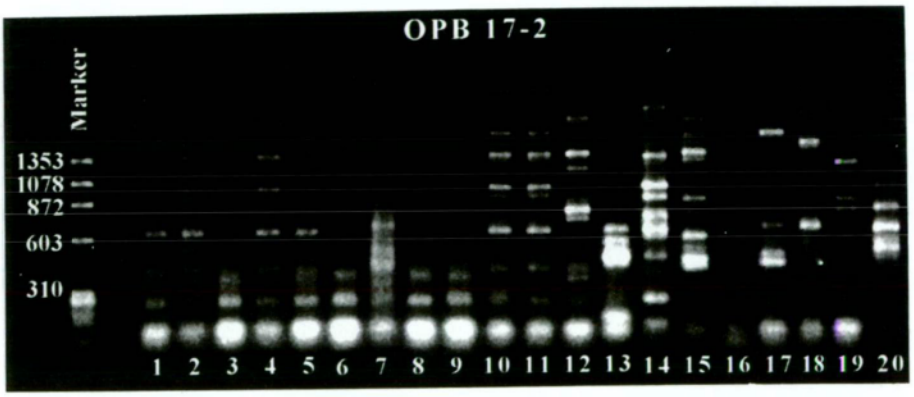



**Figure 4.2.25** Diagrammatic representation of the reproducible bands with primer OPB 12 (2nd Cycle), showing the *Streptococcus* sp. biovar 1 strains (lanes 1 to 10) *Enterococcus seriolicida* (ATCC 49156) (lane 11) and *Lactococcus garvieae* (LMG 8893) (lane 11, gel 2)



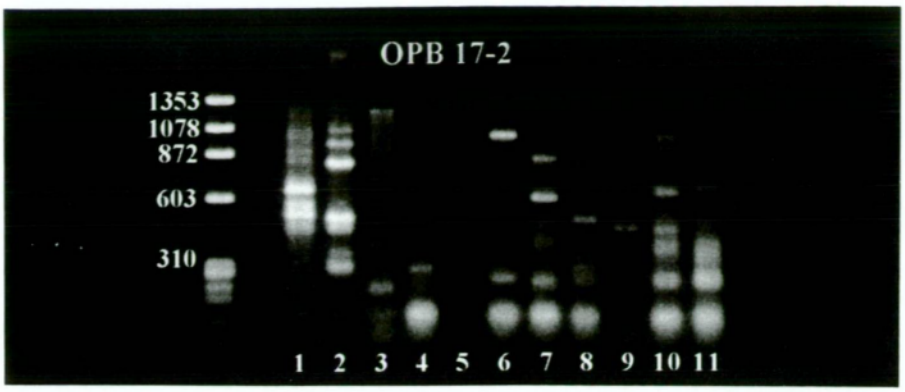

| Lane | Sample | Lane | Sample | Lane | Sample | Lane | Sample |
|---|---|---|---|---|---|---|---|
| 1 | MP6 | 6 | MP21 | 11 | UT3B | 16 | UT4C |
| 2 | MP9 | 7 | MP28 | 12 | UT6B | 17 | UT7E |
| 3 | MP16 | 8 | MP36 | 13 | UT8B | 18 | UT9F |
| 4 | MP17 | 9 | MP38 | 14 | UT1C | 19 | UT2G |
| 5 | MP20 | 10 | MP39 | 15 | UT2C | 20 | UT2I |

**Figure 4.2.26** Samples tested with primer OPB 17 (1st cycle)




| Lane | Sample | Lane | Sample | Lane | Sample | Lane | Sample |
|---|---|---|---|---|---|---|---|
| 1 | MP6 | 6 | MP21 | 11 | UT3B | 16 | UT4C |
| 2 | MP9 | 7 | MP28 | 12 | UT6B | 17 | UT1D |
| 3 | MP16 | 8 | MP36 | 13 | UT8B | 18 | UT2D |
| 4 | MP17 | 9 | MP38 | 14 | UT1C | 19 | UT4D |
| 5 | MP20 | 10 | MP39 | 15 | UT2C | 20 | UT8D |

**Figure 4.2.27** Samples tested with primer OPB 17 (2nd cycle)A




| Lane | Sample | Lane | Sample | Lane | Sample |
|---|---|---|---|---|---|
| 1 | UT9D | 6 | UT9F | 11 | MP/LG |
| 2 | UT4E | 7 | UT2G | | |
| 3 | UT7E | 8 | UT9H | | |
| 4 | UT9E | 9 | UT1I | | |
| 5 | UT8F | 10 | UT2I | | |

**Figure 4.2.28** Samples tested with primer OPB17 (2nd cycle)B

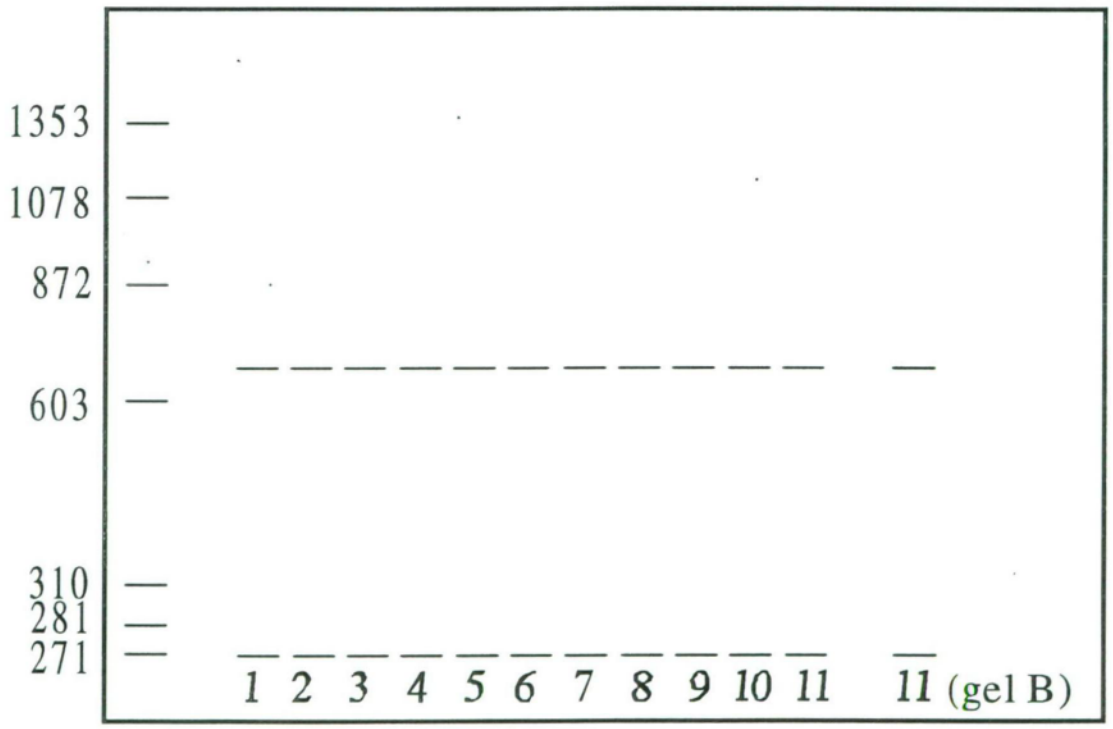
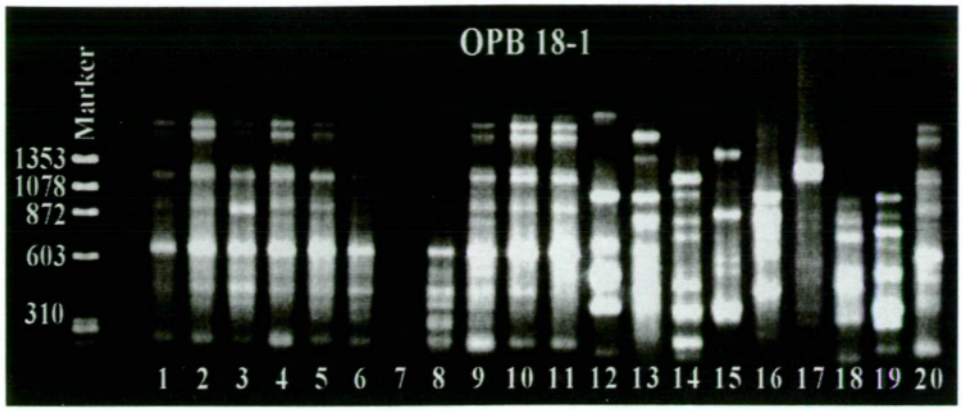
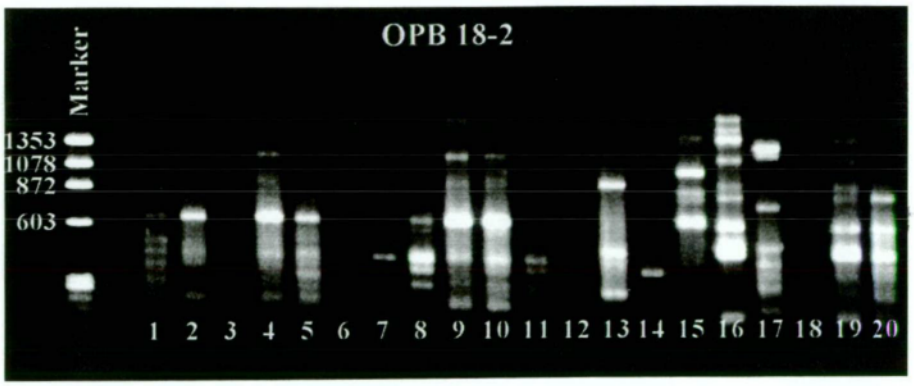



**Figure 4.2.29** Diagrammatic representation of the reproducible bands with primer OPB 17 (2nd Cycle), showing the *Streptococcus* sp. biovar 1 strains (lanes 1 to 10) *Enterococcus seriolicida* (ATCC 49156) (lane 11) and *Lactococcus garvieae* (LMG 8893) (lane 11, gel 2)




| Lane | Sample | Lane | Sample | Lane | Sample | Lane | Sample |
|---|---|---|---|---|---|---|---|
| 1 | MPL6 | 6 | MPL21 | 11 | UT3B | 16 | UT4C |
| 2 | MPL9 | 7 | MPL28 | 12 | UT6B | 17 | UT7E |
| 3 | MPL16 | 8 | MPL36 | 13 | UT8B | 18 | UT9F |
| 4 | MPL17 | 9 | MPL38 | 14 | UT1C | 19 | UT2G |
| 5 | MPL20 | 10 | MPL39 | 15 | UT2C | 20 | UT2I |

**Figure 4.2.30** Samples tested with primer OPB 18 (1st cycle)

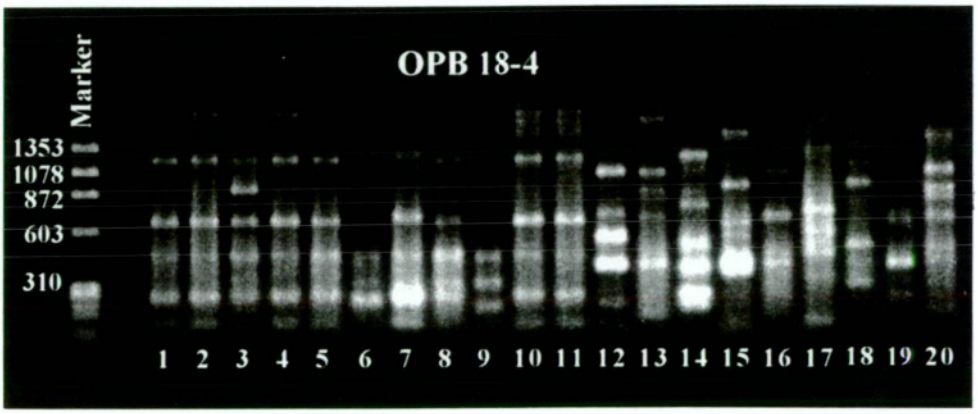



| Lane | Sample | Lane | Sample | Lane | Sample | Lane | Sample |
|---|---|---|---|---|---|---|---|
| 1 | MPL6 | 6 | MPL21 | 11 | UT4C | 16 | UT9D |
| 2 | MPL9 | 7 | MPL36 | 12 | UT1D | 17 | UT9E |
| 3 | MPL16 | 8 | MPL38 | 13 | UT2D | 18 | UT8F |
| 4 | MPL17 | 9 | MPL39 | 14 | UT4D | 19 | UT9H |
| 5 | MPL20 | 10 | UT2I | 15 | UT8D | 20 | UT1I |

**Figure 4.2.31** Samples tested with primer OPB 18 (2nd cycle)

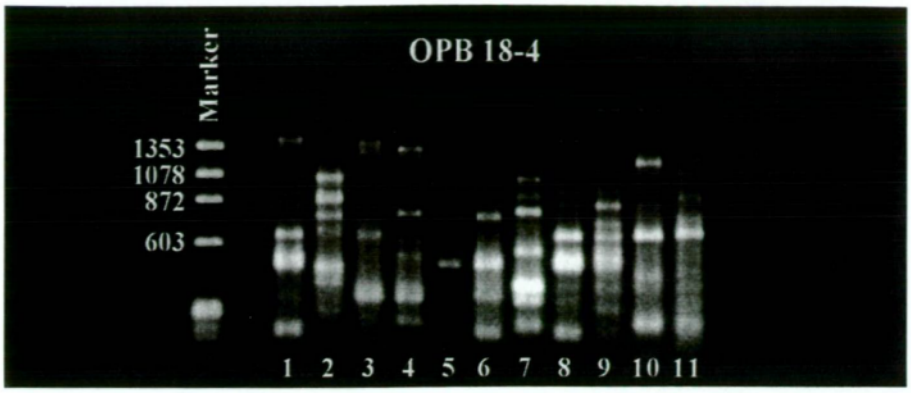

| Lane | Sample | Lane | Sample | Lane | Sample | Lane | Sample |
|---|---|---|---|---|---|---|---|
| 1 | MPL6 | 6 | MPL21 | 11 | UT4C | 16 | UT9D |
| 2 | MPL9 | 7 | MPL36 | 12 | UT1D | 17 | UT9E |
| 3 | MPL16 | 8 | MPL38 | 13 | UT2D | 18 | UT8F |
| 4 | MPL17 | 9 | MPL39 | 14 | UT4D | 19 | UT9H |
| 5 | MPL20 | 10 | UT2I | 15 | UT8D | 20 | UT1I |

**Figure 4.2.32** Samples tested with primer OPB 18 (3rd cycle)




| Lane | Sample | Lane | Sample | Lane | Sample | Lane | Sample |
|---|---|---|---|---|---|---|---|
| 1 | MPL6 | 6 | MPL21 | 11 | UT3B | 16 | UT4C |
| 2 | MPL9 | 7 | MPL28 | 12 | UT6B | 17 | UT1D |
| 3 | MPL16 | 8 | MPL36 | 13 | UT8B | 18 | UT2D |
| 4 | MPL17 | 9 | MPL38 | 14 | UT1C | 19 | UT4D |
| 5 | MPL20 | 10 | MPL39 | 15 | UT2C | 20 | UT8D |

**Figure 4.2.33** Samples tested with primer OPB 18 (4th cycle)A




| Lane | Sample | Lane | Sample | Lane | Sample |
|---|---|---|---|---|---|
| 1 | UT9D | 6 | UT9F | 11 | MP/LG1 |
| 2 | UT4E | 7 | UT2G | | |
| 3 | UT7E | 8 | UT9H | | |
| 4 | UT9E | 9 | UT1I | | |
| 5 | UT8F | 10 | UT2I | | |

**Figure 4.2.34** Samples tested with primer OPB18 (4th cycle)B


**Figure 4.2.35** Diagrammatic representation of the reproducible bands with primer OPB 18 (4th Cycle), showing the *Streptococcus* sp. biovar 1 strains (lanes 1 to 10) *Enterococcus seriolicida* (ATCC 49156) (lane 11) and *Lactococcus garvieae* (LMG 8893) (lane 11, gel B)

**Table 4.2.3 Results of 20 primers used in the RAPD assay**

| PRIMER CODE | 5' to 3' | REPRODUCIBLE BANDS | SIZE IN BASE PAIRS | NO. OF REPLICATES PERFORMED |
|---|---|---|---|---|
| OPB-01 | GTTTCGCTCC | | | |
| OPB-02 | TGATCCCTGG | | | |
| OPB-03 | CATCCCCCTG | | | |
| OPB-04 | GGACTGGAGT | | | |
| OPB-05 | TGCGCCCTTC | | | |
| OPB-06 | TGCTCTGCCC | | | |
| OPB-07 | GGTGACGCAG | 5 | 850/740/450/355 300 | 2 |
| OPB-08 | GTCCACACGG | | | |
| OPB-09 | TGGGGGACTC | 5 | 1722/1648/1410/ 1110/640 | 3 |
| OPB-10 | CTGCTGGGAC | 1 | 650 | 3 |
| OPB-11 | GTAGACCCGT | | | |
| OPB-12 | CCTTGACGCA | 3 | 610/466/407 | 2 |
| OPB-13 | TTCCCCCGCT | | | |
| OPB-14 | TCCGCTCTGG | | | |
| OPB-15 | GGAGGGTGTT | | | |
| OPB-16 | TTTGCCCGGA | | | |
| OPB-17 | AGGGAACGAG | 2 | 655/275 | 2 |
| OPB-18 | CCACAGCAGT | 6 | 1700/1590/1208/ 930/650/260 | 4 |
| OPB-19 | ACCCCCGAAG | | | |
| OPB-20 | GGACCCTTAC | | | |

49156) with fragments of ≈ 920, 1420, 1738, and 2320bp although these fragments were not visible for *Lactococcus garvieae* (LMG 8893). Primer OPB 18 gave clearly reproducible fingerprints for all *Streptococcus* sp. biovar 1 strains in cycle or replicate 1 and 4, some not so clearly visible in cycle 2 and, although one of the clearly reproducible patterns was visible in cycle 3, the other bands were not. For this reason the 4$^{th}$ cycle was performed using this primer.

The difficulty in reproducing photographs taken of ethidium bromide gels is that some of the bands are not apparent that were clearly visible on the original photos of the gels could not be reproduced for the publication of this thesis. Therefore diagrammatic representations of the true readings have been included in this section for each of the six primers. The original photographs have been retained as records.

**Discussion**

There has been a great deal of interest in this technique for DNA fingerprinting since Welsh and McClelland (1990) introduced arbitrarily primed polymerase chain reaction (AP-PCR) and Williams *et al* (1990) introduced random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR). The methods have been receiving particular attention for their simplicity and requirement for genomic DNA. Typically, RAPD analysis begins by testing the ability of different primers to generate a unique and reproducible DNA-banding profile (fingerprint) (Sakallah *et al.* 1995). This is a time consuming, but essential part of the technique. The number of primers tested and found to be suitable can vary considerably. Miyata *et al.* (1995) found that three of the twelve 12-mer primers yielded clear patterns with the strain of *Aeromonas salmonicida* subsp. *salmonicida* strain they tested. Oakey *et al.* (1996) found six of the twenty 10-mer primers gave reproducible bands for the all the species of *Aeromonas* used in their research. These results and others are summarised in Table 4.2.4

**Table 4.2.4 Summary of the primers used for RAPD-PCR**

| Species | Primers tested | Type of primer | Primers used | Author |
|---|---|---|---|---|
| *Aeromonas salmonicida* | 12 | 12-mer | 3 | Miyata *et al.* (1995) |
| *Aeromonas* sp. | 20 | 10-mer | 6 | Oakey *et al.* (1996) |
| Lactic acid bacteria † | ND | 10-mer | 1 | Cocconcelli *et al.* (1995) |
| *Legionella pneumophilia* | ND | 10-mer | 1 | Sandery *et al.* (1994) |
| *Listeria* sp. | 200 | 10-mer | 20 | Farber & Addison (1994) |
| *Listeria monocytogenes* | 1 | 10-mer | 1 | Black *et al* (1995) |
| *Listeria monocytogenes* | 11 †† | 19-mer | 1 | Niederhauser *et al.* (1994) |
| *Listeria monocytogenes* | 40 | 10-mer | 1 | Lawrence *et al.* (1993) |

ND No data

† *Lactobacillus acidophilus, helveticus, casei, reuteri* and *plantarum, Enterococcus faecalis* and *faecium and Streptococcus thermophilus*

†† Primers of different lengths were tested

A great deal of work has been done using RAPD-PCR for the identification of *Listeria* sp., especially *Listeria monocytogenes*. Farber & Addison (1994) found that, when crudely isolated DNA from *Listeria monocytogenes* strains and three 10-mer random primers were used for the RAPD analysis of these strains, distinguishable, reproducible banding profiles were obtained. They also found that RAPD was capable

of distinguishing between *Listeria* species and also between serovars within a species. Black *et al.* (1995) also found that one of the 10-mer primers used with the different *Listeria* species, including *Listeria monocytogenes*, gave RAPD fragment fingerprints that were quite distinct for the different species and serotypes. Niederhauser et al. (1994) demonstrated that a single oligonucleotide primer, under well-defined and evaluated conditions, could be used with the RAPD technique to differentiate between strains and species of *Listeria*.

*Aeromonas* species have also been intensively investigated using the RAPD technique. Miyata *et al.* (1995) demonstrated consistent profiles of *Aeromonas salmonicida* subsp. *salmonicida*, indicating their genomic homogeneity and confirming the usefulness of RAPD for the rapid identification of this pathogen. Oakey *et al.* (1996) used RAPD to differentiate the hybridisation groups of different species of *Aeromonas*, including *Aeromonas salmonicida* subsp. *salmonicida* and *Aeromonas hydrophila*, and found that, if stringently controlled, RAPD-PCR had the potential to be a valuable tool for the differentiation of *Aeromonas* hybridisation groups. Of the twenty primers (OPB-01 to 20) used in this experiment, six showed good reproducible fingerprints for the type strains tested. These results compare well with those of Oakey *et al.* (1996) who also found six primers to work well with her experiments on hybridisation groups of *Aeromonas* species.

All the "type strains", which included those previously known as *Streptococcus sp.* biovar 1 samples MP6, MP9, MP16, MP17 , MP20, MP21, MP28, MP36, MP38 and MP39, the Japanese isolate of *Enterococcus seriolicida* YT3, sample UT3B, and *Enterococcus seriolicida* (ATCC 49156), sample UT2I and the one isolate of *Lactococcus garvieae* tested (LMG 8893) sample MP/LG, showed some similar fingerprints with six of the primers (OPBs 7, 9, 10, 12, 17 and 18) suggesting that they are, indeed, all the same species. None of the environmental isolates, which were selected for their phenotypic similarity to the type strains, (see Table 4.2.1 and Figures 4.2.8 to 4.2.10, 4.2.12 to 4.2.15, 4.2.17 to 4.2.20, 4.2.22 to 4.2.24, 4.2.26 to 4.2.28 and 4.2.30 to 4.2.34) were found to have fingerprints similar to the "type strains". Not surprisingly, each of the six different primers gave totally different fingerprints. They also gave different numbers of reproducible bands within the fingerprints for

some of the strains. Although the strains tested could be seen to be the same, not all of them always displayed the entire fingerprint in each cycle probably due to the fragment not being amplified in that sample during that particular cycle.
The entire fingerprint was not always produced or visible in all cycles because the concentration of DNA may not have been sufficient or in some cases the DNA may have denatured e.g. (MP21). Fresh DNA was prepared for succeeding cycles and the fingerprints were then visible. Because the test randomly amplifies DNA and there is low stringency, it is possible that a rogue fragment can be amplified but this occurrence was rare. The reason for repeating the test at least twice is that it is necessary to establish that the bands are reproducible. There are times when bands occur that are not reproducible and these bands are not taken into consideration when determining the similarity of the isolates.

Oakey *et al* (1995) also suggested that this lack of reproducibility may also be attributed to the low stringency annealing conditions of the primer to the template, where the slightly misprimed fragments may be less reproducible than those fragments formed where the primer is totally complementary to the priming site.

Some of the problems that may be associated with technique include potential contamination of the DNA with the ethanol used in the extraction process affecting the reproducibility of the technique (Micheli *et al.* 1994), and the type of thermocycler and brand of *Taq* used also affecting the reproducibility of the fingerprints (Meunier & Grimont, 1993). Obviously the best way to solve the problem of artifactual RAPD bands caused by experimental error is to first optimise DNA extraction and amplification protocols so that the bands are consistent across replicates (Lamboy, 1994). Hence the optimisation procedure undertaken prior to the experimentation in this work ensured that there was no artifactual errors in these experiments. The major finding of this work was that the ten strains of *Streptococcus* sp. biovar 1, *Enterococcus seriolicida* (ATCC 49156) *Enterococcus seriolicida Enterococcus seriolicida* (YT3) and *Lactococcus garvieae* (LMG 8893) are genotypically similar.

# *CHAPTER FIVE:* COMPARATIVE STUDIES OF STRAINS OF *LACTOCOCCUS GARVIEAE* ISOLATED FROM FISH AND CATTLE

## Introduction

The aim of this project was to examine the pathogenicity of an Australian piscine isolate of *Lactococcus garvieae* in rainbow trout, and then to compare these results with the pathogenicity of an isolate from cattle. The first experiment was to establish the level of virulence of the Australian isolate, known to be pathogenic to rainbow trout, and, also, to examine some of the tissues from these fish both microbiologically and histopathologically.

In experiment two, a strain of *Lactococcus garvieae* isolated from a case of bovine mastitis in Tasmania was used for *in vivo* pathogenicity studies in rainbow trout. This study entailed comparing the passage the organism by a technique of direct tissue transfer (infected kidney and/or brain), as well as that of culture and reinject method. These pathogenicity studies also included the collection of some tissue samples to evaluate if there were any histopathological changes in the fish during the study.

## 5.1 Pathogenicity studies on a Tasmanian isolate of *Lactococcus garvieae*

### Methods and materials

### 5.1.1 Experimental organism

Pathogenicity studies on piscine-derived *Lactococcus garvieae* were performed by passaging one of the Tasmanian isolates twice through rainbow trout, with the intention of establishing a lethal dose in the range of 50% to 70% and then challenging the fish with the established dose. The organism randomly selected for this experiment was one of the ten Australian isolates (MP9 {88/3910-15}), which was isolated from diseased rainbow trout in Tasmania in 1988, was provided by Dr Jeremy Carson at the Department of Primary Industries and Fisheries (DPIF), Mount Pleasant Laboratories.

Ethics approval for the use of three hundred, approximately 50-100g, rainbow trout in experiment one was obtained. These fish were grouped for each procedure of

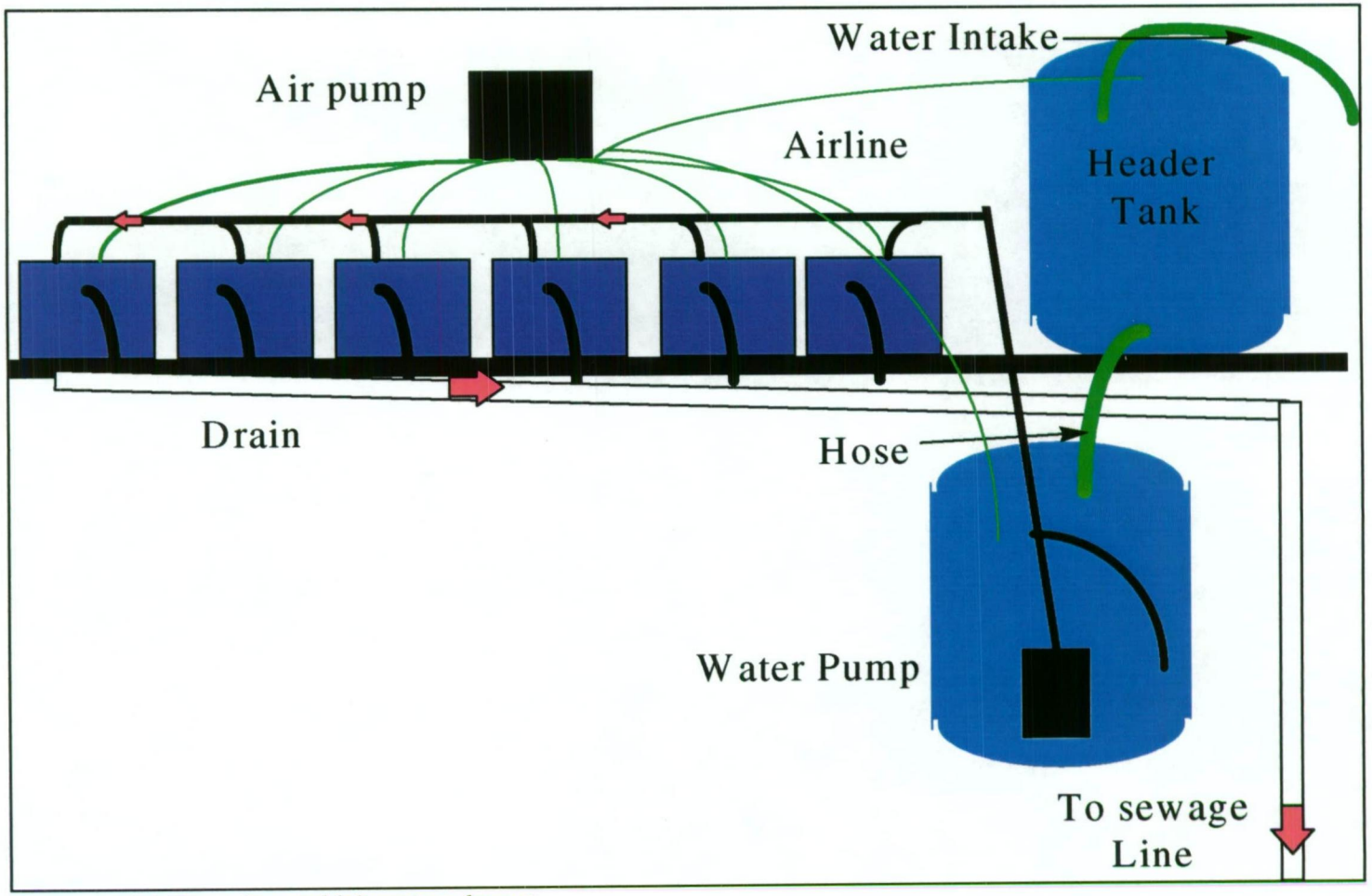



**Figure 5.1.1** Experimental laboratory layout

experiment one as shown in Table 5.1.1. Figure 5.1.2 demonstrates the procedures used in this experiment.

**Table 5.1.1 Summary of the number of fish required for each step of experiment 1**

| Step | Number of concentrations | Number of fish | Number of Organisms | Number of steps | Total |
|---|---|---|---|---|---|
| Passage | 1 | 20 | 1 | 2 | 40 |
| $LD_{50-70}$ | 3 | 40 | 1 | 2 | 240 |
| Challenge | 1 | 20 | 1 | 1 | 20 |

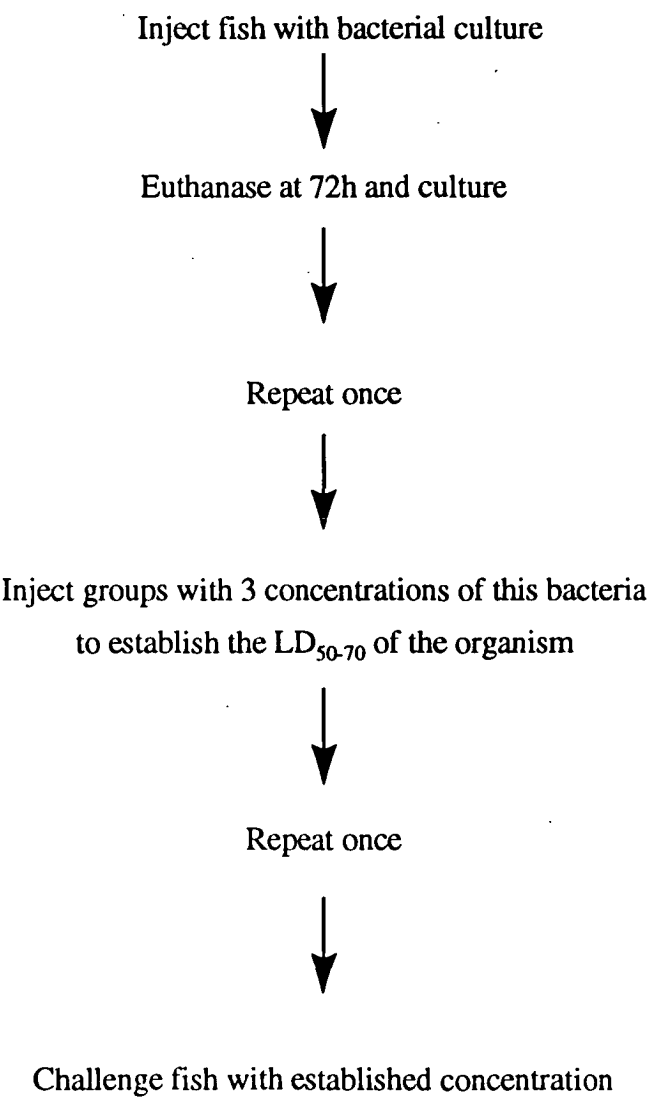



**Figure 5.1.2** Flow diagram of the passage and challenge technique

### 5.1.2 Experimental equipment

For the pathogenicity study it was necessary to construct a flow-through system in an area separate from all other facilities. Six 40L tanks were established in a laboratory (Figure 5.1.1) with fresh water entering the header tank under mains pressure via a nylex hose then draining into a holding tank via nylex hose before being pumped to the 40L tanks by an Eheim submersible pump via 13mm garden watering pipe. The header tank was fitted with a float valve to control the flow of water from the intake. The waste water was displaced from each tank by gravity into a drain which emptied into the sewage line which flowed to a plant using chlorine treatment. Each tank was given a separate air supply generated by an Eterna IV air pump which also aerated the water in the header and holding tanks through an airline and an air stone. The water temperature in the laboratory was maintained at approximately 20°C as it is known that streptococcal type disease is more likely to occur at temperatures of >18°C (Munday *et al.* 1993; Munday, 1996; Perera *et al.* 1997). This was done by heating both the air and the water in the laboratory. Water temperature, oxygen concentration, ammonia and nitrate levels were monitored by standard techniques during both the experiments and were maintained at normal levels. Faecal matter and uneaten food was siphoned out daily with a half water change.

### 5.1.3 Passage of the organism

Four groups of five (n=20) rainbow trout were placed into the 40L plastic tanks on the fresh water flow-through system. The fish were acclimated in their tanks overnight after transfer from holding tanks in the aquatic centre. Fish were fed twice daily with trout pellets.

#### 5.1.3.1 Preparation of bacterial antigen for injection

An overnight broth culture of the isolate MP9 in Brain Heart Infusion (BHI) broth (Oxoid CM225) was centrifuged, washed three times with sterile phosphate-buffered-saline (PBS) and reconstituted to the original volume with sterile PBS. The absorbance of the suspension was measured at 550nm on a Shimadzu UV-1201 spectrophotometer The suspension was then diluted 1 in 100, 1 in 1,000 and 1 in 10,000 and a standard spread plate colony count was performed to calculate the number of bacteria in the inoculum. Data already to hand on the approximate

concentration of the bacteria needed to infect the fish (Akhlaghi, 1994) were used to select the concentration of bacteria to be used in the passage. After each passage the kidney tissue was cultured, the colonies of the culture prepared as before and then reinjected into the next batch of fish. The number of bacteria used in both passages was calculated by the plate count method (Appendix D).

#### 5.1.3.2 Injection procedure

All fish were anaesthetised in benzocaine (50ppm) and two groups of five fish (n=10) were injected intraperitoneally with 0.1mL of the bacterial preparation and two groups five fish (n=10) were injected with fish physiological saline (FPS) as a control. After injection the fish were returned to their respective tanks to recover.

### 5.1.4 Re-isolation of inoculant

Tanks were inspected 12 hourly for 72h and all mortalities removed and any moribund fish euthanased in 100ppm of benzocaine and taken to the laboratory for sampling.

#### 5.1.4.1 Post-mortem techniques

During this experiment, including the passage and $LD_{50}$ sections, different types of tissue were collected from euthanased moribund fish, for microbiological and histopathological examination. The types of tissue used for these techniques is summarised in Table 5.1.2

**Table 5.1.2 Types of tissue collected for histology and/or culture**

| | Histology | Culture |
|---|---|---|
| Eye | Yes | Yes |
| Brain | Yes | Yes |
| Gill | Yes | No |
| Heart | Yes | Yes |
| Liver | Yes | No |
| Ant. Kidney | Yes | Yes |
| Post. Kidney | Yes | No |
| Spleen | Yes | No |
| Stomach | Yes | No |
| Intestine | Yes | No |
| Skin/muscle | Yes | No |

To prevent contamination of isolates with external organisms, sterile procedures were always used when dissecting fish for bacterial isolation. The excess mucus was first wiped from the fish with a clean paper towel. The area for incision was then wiped or sprayed with 70% ethyl alcohol. For all dissections, instruments were placed in 70% alcohol and then flamed using a Bunsen burner.

The viscera were exposed by cutting through the wall of the abdomen from the vent to the operculum (Figure 5.1.3). Using sterile forceps and fine scissors the heart was removed into a sterile petri dish and blood for culture aseptically drawn with a 1mL syringe using a 26 gauge needle. The anterior kidney was best accessed by making a deep transverse cut in front of the dorsal fin and continuing the incision through the kidney and swim bladder, parting the tissue and sampling the kidney through the opening with a sterile swab or pipette or a flamed loop. Care was taken not to cut through the intestine. The brain was sampled in a similar manner except that the incision was made just behind the eyes. When the brain samples had been collected to the anterior surface of the eye was pierced with a sterile glass pipette and fluid removed for culture. The eye was then excised along with some of the optic nerve fibre and muscles.

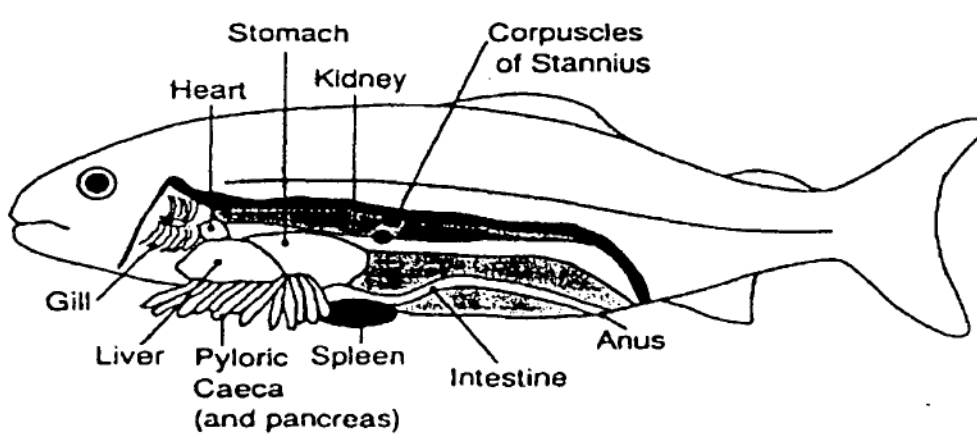



**Figure 5.1.3** General location of internal organs of a typical fish from an abdominal incision approach (Whitman & MacNear, 1994)

Although kidney is the site of choice for culturing septicaemic fish, the brain and eye are also useful sites as this organism also has a predilection for these tissue (Kusuda & Kimura, 1978; Kimura & Kusuda, 1979). After sampling of the tissue for the microbiological examination, tissue samples from the different organs (Table 5.1.2) were placed into 10% neutral-buffered-formalin for further processing.

### 5.1.4.2 Culture of post mortem samples

Samples were pipetted, swabbed or plated onto HBA (Appendix A7) as well as into the selective enrichment broth BSSB (Appendix A2) and incubated aerobically overnight at 35°C. Plates were examined for typical colonies and the growth was semi-quantified. If no growth was found on the primary culture then the selective enrichment broth was subcultured onto HBA and MSSA (Appendix A3). These plates were then examined for typical colonies.

### 5.1.5 Experimental infection

After the organism *Lactococcus garvieae* (MP9) was passaged twice through the rainbow trout, 10-fold dilutions were prepared from the growth of the final passage. The growth of an overnight broth culture in BHI broth was centrifuged and washed three times in PBS and reconstituted to the original volume with sterile PBS and absorbance adjusted to 0.33 at 550nm. This was then diluted 10 fold (1 in 100, 1 in 1,000 and 1 in 10,000) for injection. A plate colony count was performed to confirm the number of bacteria in the inoculum.

The three groups of fish (n=10) were injected with 0.1mL of these dilutions to establish which dilution of the bacteria would produce 50-70% mortality based on the method of Reed and Muench (1938). This experiment was undertaken in replicate with three groups of fish (five per tank) for the three concentrations of the bacterium. The concentrations used were approximately $10^3$, $10^4$ and $10^5$ cfu/mL. A similar control group of 10 fish were injected with sterile FPS and held under the same conditions in two additional tanks. Once the approximate $LD_{50-70}$ had been established, ten fish (five per tank) were then challenged with the established concentration of bacteria.

### 5.1.6 Histopathology

Tissue samples were taken from eye, brain, gill, heart, liver, anterior kidney, posterior kidney, spleen, stomach, intestine and skin/muscle for histology. The skin/muscle sample included tissue from the lateral line. The samples were fixed in 10% neutral-buffered-formalin overnight and then processed by standard procedures with a Tissue Tec II processor. The tissue was then wax-embedded and four sections cut at 3μ from each sample for staining with haematoxylin & eosin stain (H & E), Gram stain, Giemsa stain and periodic acid-Schiff (PAS) (Bancroft & Stevens, 1996). The slides were then examined for the presence of bacteria or typical inflammation in the tissue.

## Results

### 5.1.3 Passage of the organism

During the two *in vivo* passages, the bacteria were seen to increase in virulence, distinguished by the observation that more fish were succumbing earlier to the same dose of bacteria, which was between $10^5$ to $10^6$ cfu/mL with 20% morbidity and mortality after the first passage and 50% after the second passage. There were no signs of disease or mortality seen in the control group.

### 5.1.4 Re-isolation of inoculant

The organism was readily reisolated from most of the tissue samples of the mortalities, especially the kidney as summarised in Table 5.1.3. The results of the semi-quantification of growth upon re-isolation of the organism showed quite clearly that, as expected, the anterior kidney yielded the greatest number of organisms followed by the brain. The eye and heart cultures mostly yielded little or no growth. For future experiments the anterior kidney was, therefore, the chosen site for the re-isolation of the organism.

### 5.1.5 Experimental infection

Data from the passaging of the organism was used to decide the three concentration of bacteria to be used to establish a lethal dose in the range of 50% to 70%. The three 10 fold dilutions gave the following results averaged over both replicates as shown in Table 5.1.4

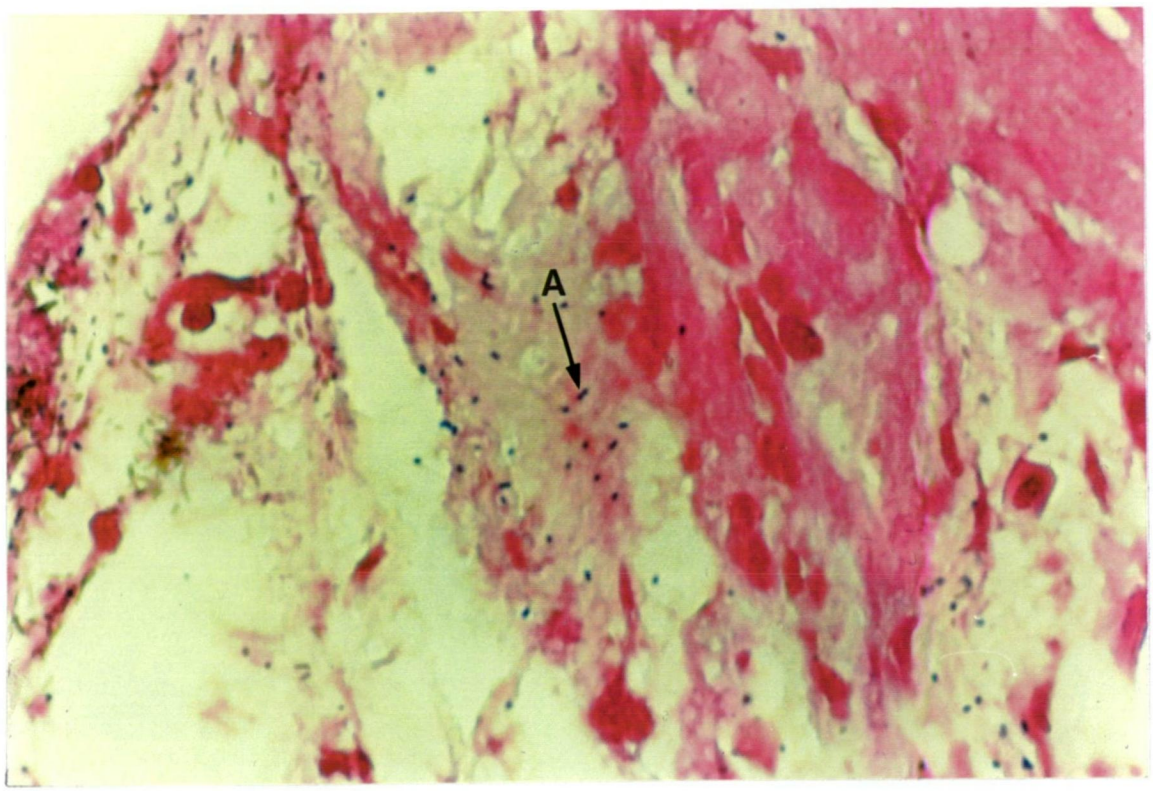



**Figure 5.1.4** Gram stain of brain tissue showing Gram-positive cocci **A** x1000

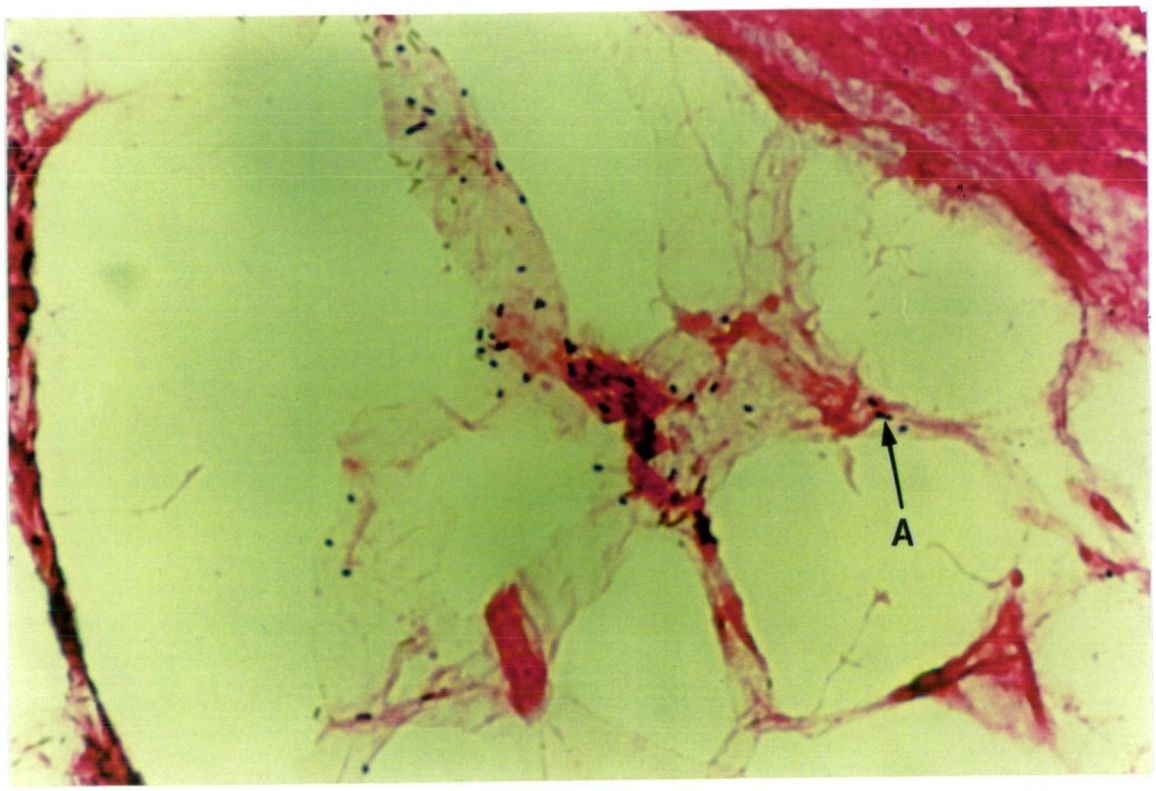



**Figure 5.1.5** Gram stain of the meninges showing Gram-positive cocci **A** x1000

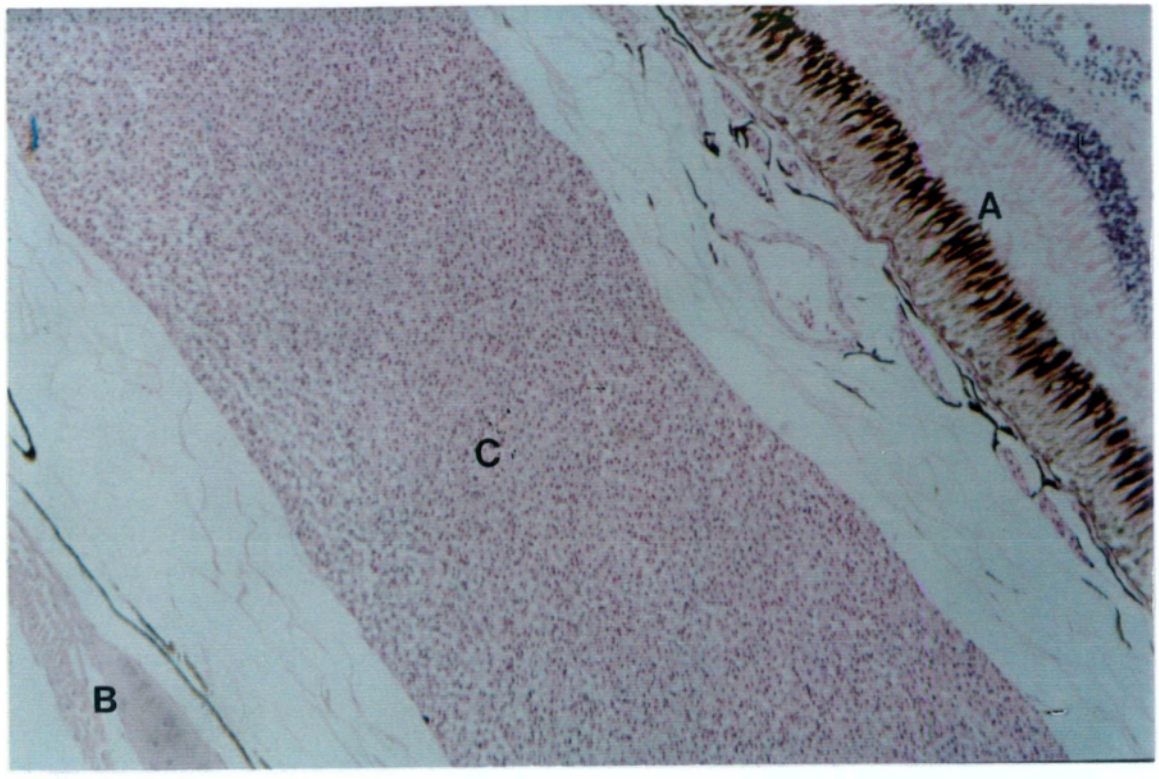



**Figure 5.1.6** H&E stain of the eye showing separation of the retina **A** and sclera **B** with a large haematoma **C**

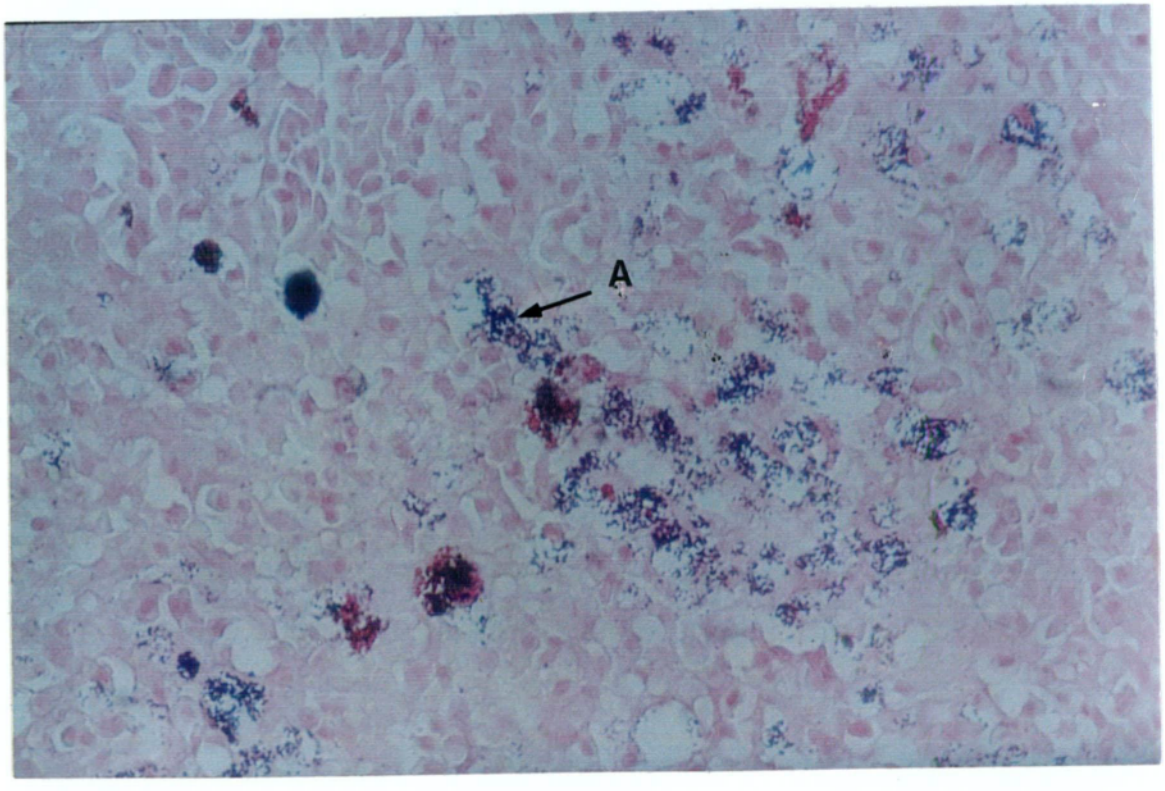



**Figure 5.1.7** Gram stain of spleen tissue showing Gram-positive cocci **A** x100

**Table 5.1.3 Semi-quantification of growth from different tissue samples**

| Fish No. | Eye | Brain | Heart | Kidney |
|---|---|---|---|---|
| 1 | LG | HG | NG | HG |
| 2 | LG | MG | NG | HG |
| 3 | MG | HG | NG | HG |
| 4 | NG | MG | LG | HG |
| 5 | NG | HG | LG | HG |
| 6 | NG | LG | NG | HG |
| 7 | LG | MG | NG | HG |
| 8 | NG | MG | NG | HG |
| 9 | NG | NG | NG | NG |
| 10 | NG | LG | NG | MG |
| 11 | LG | HG | LG | HG |
| 12 | LG | HG | NG | HG |
| 13 | NG | MG | NG | HG |
| 14 | NG | MG | NG | HG |
| 15 | LG | MG | NG | HG |
| 16 | MG | MG | NG | HG |
| 17 | NG | HG | NG | MG |
| 18 | NG | NG | NG | NG |
| 19 | NG | NG | NG | LG |
| 20 | NG | NG | NG | MG |

NG no growth
LG light growth
MG moderate growth
HG heavy growth

**Table 5.1.4 Results of the passaging of the Tasmanian isolate of *Lactococcus garvieae* in replicate**

| Dilution | Average CFU/0.1mL | Average % Mortality |
|---|---|---|
| 1:100 | $2 - 4 \times 10^4$ | 75 |
| 1:1000 | $2 - 5 \times 10^3$ | 47 |
| 1:10,000 | $6 \times 10^2$ | 22.5 |

The challenge was performed by injecting the 1:1000 dilution on 2 groups of 5 fish (n=10) with control groups of 5 fish per tank (n=10) being injected with FPS under the same conditions. The 1:1000 dilution gave a viable cell count of $1.7 \times 10^4$cfu/mL and resulted in the mortality of 6 of the 10 fish ($LD_{60}$). None of the control fish died.

### 5.1.6 Clinical disease and histopathology

Overt signs of disease in moribund fish included swimming sluggishly with a spiralling motion, curved body, anorexia, darkening of the skin, distended abdomen and

exophthalmia in one or both eyes. Internal signs of the disease mainly included haemorrhaging and fluid in and around the internal organs and sometimes enlargement and darkening of the spleen. As these fish had been experimentally infected with a high dose, signs were mainly those of acute infection rather than chronic.

After making a vertical incision through the skull behind the eyes there was visual evidence of haemorrhage around the brain. Gram stain of the brain and meninges showed numerous Gram-positive cocci present (Figures 5.1.4 and 5.1.5). The eyes often appeared cloudy with haemorrhagic spots and upon removal there was signs of haemorrhage in the anterior chamber. Histologically, the sclera was often separated from the retina with haemorrhaging and/or inflammation in the interstice (Figure 5.1.6).

Gram-positive bacteria were seen throughout the spleen (Figure 5.1.7). The gills showed swelling in the primary lamellae due to the infiltration of erythrocytes and some macrophages, while the secondary lamellae were often distorted and fused. Also, PAS stain showed greatly increased mucus on the gill tissue compared to normal gill tissue. The stomach and intestine showed degeneration of the mucosal folds and large numbers of erythrocytes in the lumen resulting from haemorrhaging. The PAS stain of the intestinal tract showed some sloughing of the mucosa. The kidney was liquid and congested and showed mild degeneration of the renal tubules. The overall picture was that of a bacteraemia or septicaemia with meningitis one of the salient features.

**Discussion**

Experimental infection of streptococcosis (or enterococcosis or lactococcosis) has been performed on a number of different species of fish world-wide including rainbow trout (Boomker *et al.* 1979; Munday *et al.* 1993; Ghittino *et al*, 1995; Akhlaghi, 1994), tilapia (Eldar *et al.* 1995a; Chang & Plumb, 1996; Perera *et al.* 1997 ), channel catfish (Chang & Plumb, 1996), and yellowtail (Fukuda *et al.* 1997).

Boomker *et al.* (1979) experimentally infected rainbow trout using three experiments with large doses of *Streptococcus* sp. previously isolated from diseased rainbow

trout. These experiments were mainly done to study the differences between the route of delivery and to also study the pathology of the disease. The bacterium was not passaged and an $LD_{50}$ was not established. Munday *et al.* (1993) studied the pathogenicity of *Streptococcus* sp. biovar 1 (*Lactococcus garvieae*) in rainbow trout in comparison to Atlantic salmon and brown trout in two experiments. The first experiment showed that the mortality for rainbow trout was greater the higher the temperature (18-20°C) with an approximate $LD_{50}$ of between $2.04 \times 10^4$ to $5.46 \times 10^6$. The second experiment was performed to compare the pathogenicity to rainbow trout and brown trout, and also to compare the injection technique versus horizontal transmission. Injection gave 100% mortality in the rainbow trout compared to 0% in the brown trout and there was no clear evidence of horizontal transmission in either fish species. As a result of both these experiments, it was established the bacterium was much more pathogenic for rainbow trout than brown trout and Atlantic salmon.

Akhlaghi, (1994) reported that increasing the virulence of bacterial pathogens with animal passage was a well recognised phenomenon, especially with Gram-positive bacteria. He found that after five passages of *Streptococcus* sp. now known to be *Lactococcus garvieae* (previously *Streptococcus sp.* biovar 1) the $LD_{70}$ was reduced from $2\text{-}3 \times 10^4$cfu/mL to as low as 50cfu/mL. Similarly, in the experiment reported here, the organism showed increase virulence with each passage.

Ghittino *et al.* (1995) also examined the pathogenicity of streptococcal disease in rainbow trout with an organism believed to be closely related to *Enterococcus seriolicida* at that time. They found that the organism became highly pathogenic with an $LD_{50}$ decreasing from $10^7$cfu/mL to $10^2$cfu/mL after 4 passages.

Eldar *et al.* (1995a) increased the virulence of *Streptococcus shiloi* (now known as *Streptococcus iniae*{Eldar *et al.* 1995a}) and *Streptococcus difficile* from $LD_{50}$ $10^7$-$10^8$cfu $10^2$-$10^5$cfu after 3 passages in tilapia. The technique they used was brain to brain passage without culture plates, a technique that we were unable to perfect in our second experiment. Chang & Plumb (1996) experimentally infected tilapia and channel catfish with 3 unnamed strains of *Streptococcus* spp. isolated from diseased fish. The infectivity trial involved scraping the fishes lateral body surface and the

immersing them in approximately 2.4 x $10^7$cfu/mL of the different strains of bacteria for 10min. The eventual mortality of tilapia was 0-40% and 13-37% in the channel catfish but no attempt was made to establish an $LC_{50}$ in this experiment.

In summary, all these studies indicate the ease with which these organisms increase virulence with passage and suggest that this type of behaviour is likely to be significant in the evolution of outbreaks of streptococcosis (enterococcosis and lactococcosis). Because the cultures were washed three times in sterile PBS before injection this excluded the possibility of extracellular products or exotoxins contributing to the virulence. It must be remembered, however, that such biological experiments vary amongst themselves and therefore direct numerical comparisons can only be made within the one set of observations. Although eye, brain, heart and kidney samples were cultured for the re-isolation of the injected bacterium, it was found that, as expected, the kidney was the ideal site for re-isolation, a fact that confirmed reports by Kusuda & Kimura (1978) and Kimura & Kusuda (1979) that kidney tissue was the most likely site to reisolate the organism.

The histopathology was found to be similar to other reports of *Streptococcus* infection in fish. Chang & Plumb (1996) found that there was infiltration of fatty tissue into areas of severe inflammation and breakdown of tissue consistent with septicaemia after experimental infection in tilapia and channel catfish. They also found exophthalmia occurring in tilapia also associated with ocular opacity. Although they found that the disease was less severe in the channel catfish, there was inflammatory exudate in the brain with increased macrophages, lymphocytes and erythrocytes. Also, lesions in the lens of the eye showed lenticular fibre disruption and separation in these fish.

Boomker *et al.* (1979) experimentally infected rainbow trout with *Streptococcus* sp. isolated from an outbreak of streptococcosis. Clinical signs of the disease were lethargy, anorexia, exophthalmia and opacity of one or both eyes, distended abdomen and some dark pigmentation of the skin. The bacterium was isolated from the spleen liver and kidneys of all the trout. Gross pathology showed enlarged spleens and congested kidneys. Histopathology showed fatty degeneration of the liver, congested

spleen, haemorrhagic kidneys, heart muscle degeneration and the eye showed detachment of the sclera from the retina and choroid with haematomas in between consistent with our findings (see Figure 5.1.6). Small petechial haemorrhages were seen in the gill with thrombi in the vessels of the gill filaments, some of which were fused.

Baya *et al.* (1990) examined infected fish from Chesapeake Bay, including bluefish, striped bass and sea trout. Exophthalmia was a common feature of the disease in striped bass, while the bluefish and sea trout mainly exhibited corneal opacity only. The abdominal cavity of all three species was distended and filled with reddish-yellow fluid and the spleens were enlarged and dark. Microscopically, there was evidence of chronic inflammation of the eye, optic nerve and brain. In the spleen there was a depletion of lymphocytic and lymphoblastic cells. Gram stains of smears from kidney, spleen, liver and brain tissues showed large numbers of streptococcus-like organisms.

Nieto *et al.* (1995) examined diseased turbot infected either naturally or experimentally by *Enterococcus* sp. by taking samples from skin, muscle, gills, eyes, intestine, liver, kidney, spleen, heart and brain for bacteriological and histopathological studies. These were processed and stained with H&E, PAS and Gram stains. At necroscopy, gross findings were uni- or bilateral exophthalmia, purulent exudate in the abdominal cavity, mucopurulent exudate in the cranial cavity and muscle haemorrhages. Histopathological findings included lesions associated with acute branchitis, with large numbers of neutrophils within the venous sinuses of the secondary lamallae, with lamaellar fusion, haemorrhages and mucous metaplasia. In the eye there was evidence of haemorrhages and neutrophil infiltration which caused the exophthalmia. Gram-positive cocci were seen in the spleen, including ellipsoids and melaomacrophage areas. Although Gram-positive cocci were found in the interstitial macrophages of the kidney, no lesions were observed in the tubules. Vacuolation and fatty degeneration were observed in the liver.

Kusuda & Salati (1993) described the pathology of *Enterococcus seriolicida* infection in yellowtail, sea bream and flounder as bilateral exophthalmia, petechiae on the inside walls of the operculum and congestion and haemorrhaging of the intestine, liver and

spleen. Histopathological features included degeneration of the tissues and necrosis in many organs including the heart, gill, skin, spleen and eye.

From this it can be seen that all the authors found many similarities in the signs of streptococcal disease, despite the variation in the organisms involved and the species of fish affected. Most of the findings match those of this experiment, especially those of Boomker *et al.* (1979) who also studied the disease in rainbow trout. This, therefore, confirms the findings of this experiment, that overwhelming disease can be produced in rainbow trout with very low doses of *Lactococcus garvieae* with signs characteristic with other cases of streptococcosis (especially lactococcosis) elsewhere in the world.

## 5.2 Pathogenicity studies on a bovine isolate of *Lactococcus garvieae*

### Methods and materials

### 5.2.1 Experimental organism

*Lactococcus garvieae* strain MPL 94-4127.2 was obtained from Dr Jeremy Carson at the DPIF, Mount Pleasant Laboratories in a freeze dried form and reconstituted in BHI broth, subcultured onto HBA and incubated overnight at 35°C. This organism had been sub-cultured four times by this stage. For this experiment two methods of transferring the organism to the fish were trialed. The first method was to inject the bacterial culture and then take kidney tissue from the fish, homogenise the tissue and reinject this tissue directly into the next group of fish. The second method was as previously described in Chapter 5.1.3.

For this experiment, a renewal application was approved by the ethics committee for the use of 128, approximately 50-100g, rainbow trout, sufficient to repeat the experiment if necessary. These fish were grouped for each procedure of experiment two as shown in Table 5.2.1

### 5.2.2 Experimental equipment

For this experiment a facility that was already constructed for disease and toxicology work was utilised. This facility contained eight 250L tanks operating on a flow through freshwater system, hence larger fish could be used. Each tank was given a separate air supply generated by an Eterna IV air pump which also aerated the water

**Table 5.2.1 Summary of the number of fish required for each step of experiment two**

| Step | Number of concentrations | Number of fish | Number of Organisms | Number of steps | Total |
|---|---|---|---|---|---|
| Passage 1 † | 1 | 8 | 1 | 4 | 32 |
| Passage 2 ‡ | 1 | 8 | 1 | 4 | 32 |

† Tissue transfer technique (Group A)

‡ Re-isolation technique (Group B)

in the header and holding tanks through airline and an air stone. The water temperature in the laboratory was maintained at approximately 20°C by heating both the air in the laboratory and the water in the tanks with aquarium heaters.

### 5.2.3 Passage of the organism

The rainbow trout were divided into groups of four in four (n=16) separate 250L plastic tanks on a freshwater flow-through system and classified as Group A & B. The two procedures are summarised in Figure 5.2.1

#### 5.2.3.1 Preparation of the bacterial antigen for injection

An overnight broth culture BHI of the isolate MPL 94-4127.2 was centrifuged, washed three times with sterile PBS and reconstituted to the original volume with sterile PBS. This was not diluted for injection. A plate colony count was performed to calculate the number of bacteria in the inoculum. The number of bacteria used in both passages was calculated by the plate count method.

#### 5.2.3.2 Passage of bacteria by tissue transfer (Group A)

Group A1 (n=8) was injected intraperitoneally with 0.1 mL of $10^8$ *Lactococcus garvieae* (MPL94-4127.2) culture diluted in PBS. After 72h the fish were euthanased by the standard protocol and the anterior kidney from each fish aseptically removed by dissection. The kidneys were then pooled, homogenised in the minimum amount of PBS (≈1mL) with a Polytron-Aggregate®, filtered through sterile glass wool and 0.1mL injected intraperitoneally into Group A2 (n=8) fish. The homogenate was also cultured by inoculating into enrichment broth and onto a blood agar plate and incubating aerobically overnight at 35°C. The procedure of kidney transfer was

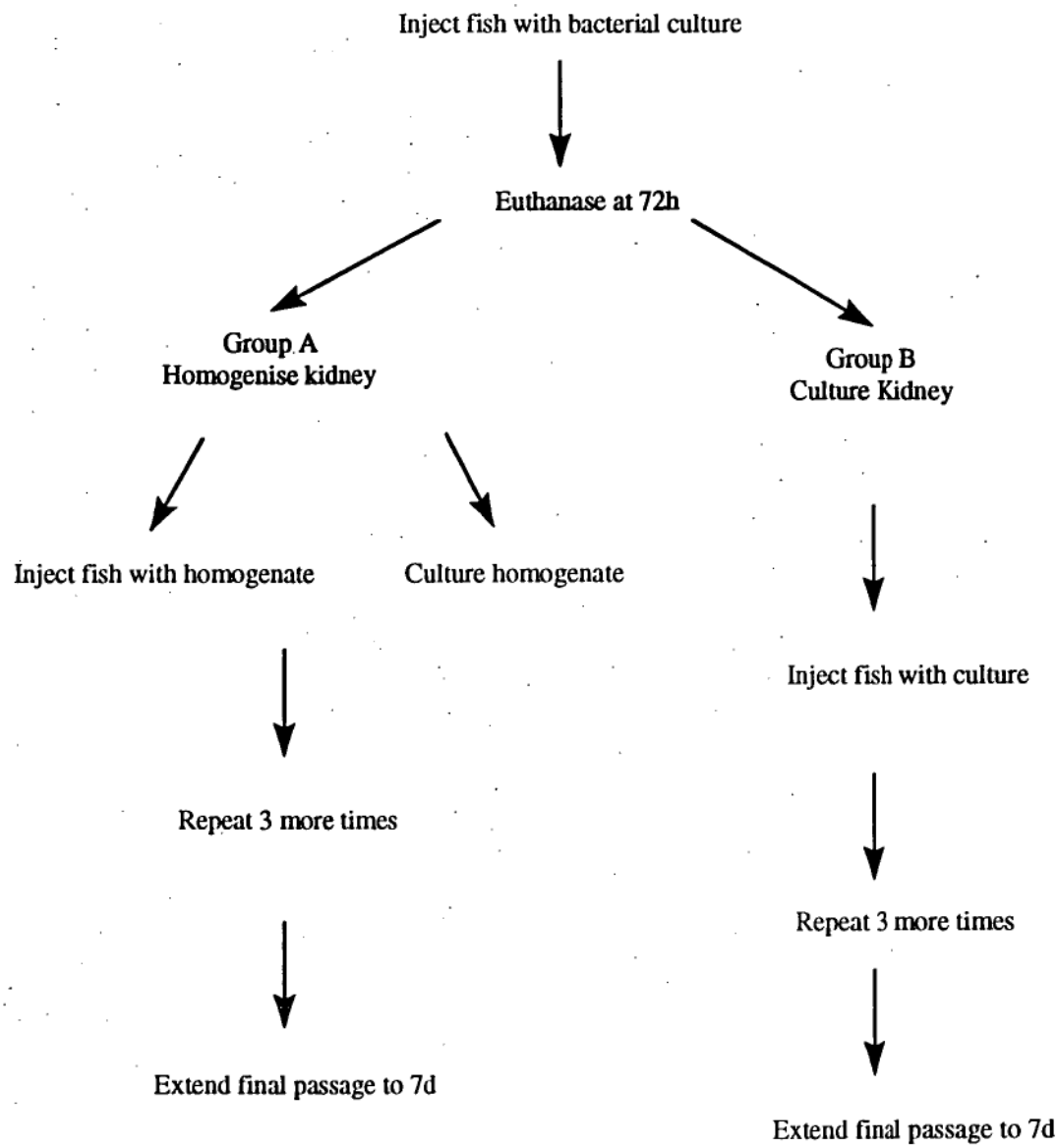



**Figure 5.2.1** Flow diagram of tissue versus culture transfer technique

repeated two more times with groups A3 & A4, where A stands for the group of fish and the number (1, 2, 3, 4) stands for the passage.

### 5.2.3.3 Passage of bacteria by culture (Group B)

Group B1 (n=8) was injected intraperitoneally with 0.1 mL of $10^8$ cfu/mL *Lactococcus garvieae* (MPL94-4127.2) culture diluted in PBS. After 72h the fish were euthanased in benzocaine and the anterior kidney from each fish cultured onto HBA and incubated overnight at 35°C. Growth from the plates was pooled into PBS to $10^8$ cfu/mL (confirmed by plate count) and 0.1mL injected into Group B2 (n=8)

fish. The procedure of culture transfer was repeated two more times with groups B3 & B4.

### 5.2.4 Re-isolation of inoculant

Samples were pipetted, swabbed or plated onto HBA as well as into an selective enrichment broth such as BSSB and incubated aerobically overnight at 35°C. Plates were examined for typical colonies and the growth was semi-quantified. If no growth was found on the primary culture, then the selective enrichment broth was subcultured, onto HBA as well as the MSSA. These plates were then examined for typical colonies.

### 5.2.5 Clinical signs and histopathology

Histopathology samples were taken from the eye with surrounding tissue and from the brain. The samples were fixed in 10% neutral-buffered formalin and then processed by standard procedure with a Tissue Tec processor. The tissue was wax-embedded and two sections cut from each sample. These sections were then stained with haematoxylin and eosin (H & E). The slides were the examined for the presence of bacteria or typical signs of disease such as inflammation in the tissue.

## Results

### 5.2.3 Passage of the organism

### 5.2.3.2 Passage of bacteria by tissue transfer (Group A)

The results of passaging *Lactococcus garvieae* by tissue transfer of homogenised kidney are summarised in Table 5.2.2.

After the second passage using the tissue transfer technique, it was noted that the organism was not being reisolated from the kidney tissue of these fish by the routine primary culture technique and was only able to be reisolated after broth enrichment. This phenomenon was further investigated using the same protocol, but sampling both kidney and brain tissue homogenates. The results of that experiment are summarised in Table 5.2.3.

**Table 5.2.2 Results of passaging *Lactococcus garvieae* with the transfer of kidney tissue (Group A)**

| | Technique | Protocol | Growth |
|---|---|---|---|
| 1st passage | Inject with culture | Euthanase at 72h | |
| | Culture kidney | Incubate | $>10^5$ cfu/mL |
| | Homogenise kidney for 2nd passage | | |
| | Culture homogenate | Incubate | $<10^4$ cfu/mL |
| 2nd passage | Inject with homogenate | Euthanase at 72h | |
| | Culture kidney | Incubate | No growth |
| | Culture kidney enrichment broth | Incubate | Growth |
| | Homogenise kidney for 3rd passage | | |
| | Culture homogenate | Incubate | No growth |
| | Culture homogenate enrichment broth | Incubate | Growth |
| 3rd passage | Inject with homogenate | Euthanase at 72h | |
| | Culture kidney | Incubate | No growth |
| | Culture kidney enrichment broth | Incubate | Growth |
| | Homogenise kidney for 4th passage | | |
| | Culture homogenate | Incubate | No growth |
| | Culture homogenate enrichment broth | Incubate | Growth |
| 4th passage | Inject with homogenate | Euthanase at 7d | |
| | Culture kidney | Incubate | No growth |
| | Culture kidney enrichment broth | Incubate | Growth |

**Table 5.2.3 Results of repeat passaging *Lactococcus garvieae* with transfer of kidney and brain tissue (Group A)**

| | Technique | Protocol | Result |
|---|---|---|---|
| 1st passage | Inject with culture | Euthanase at 72h | |
| | Culture kidney and brain | Incubate | $>10^5$ cfu/mL |
| | Homogenise tissue for 2nd passage | | |
| | Culture kidney homogenate | Incubate | $<10^4$ cfu/mL |
| | Culture brain homogenate | Incubate | No growth |
| | Culture brain homogenate enrichment | Incubate | Growth |
| 2nd passage | Inject with kidney and brain homogenate | Euthanase at 72h | |
| | Culture kidney | Incubate | $<10^4$ cfu/mL |
| | Culture brain homogenate | Incubate | No growth |
| | Culture brain homogenate enrichment | Incubate | Growth |
| | Homogenise tissue for 3rd passage | | |
| 3rd passage | Inject with kidney and brain homogenate | Euthanase at 7d | |
| | Culture kidney | Incubate | Growth |
| | Culture kidney enrichment broth | Incubate | Growth |
| | Culture brain homogenate | Incubate | No growth |
| | Culture brain homogenate enrichment | Incubate | Growth |
| | Collect eye and brain tissue for histology | | |

### 5.2.3.3 Passage of bacteria by culture (Group B)

Table 5.2.4 shows the results of passaging the bovine isolate of *Lactococcus garvieae* by sampling the kidney tissue with a swab or flamed loop and then plating out the bacteria each time onto solid media and incubating overnight at 35 °C. Then the growth from the plates emulsified in sterile PBS for reinjection for the next passage. A plate count of the injectant each time showed approximately $10^8$ cfu/mL.

### 5.2.5 Clinical signs and histopathology

Tissue samples were taken from the three groups of fish as summarised in Table 5.2.5. Some of the fish displayed exophthalmia and this fact was noted on the sample. Two of the fish from the seven day trial showed signs of exophthalmia, otherwise no other clinical signs consistent with streptococcosis were observed.

**Table 5.2.4 Results of passaging *Lactococcus garvieae* with culture re-isolation technique (Group B)**

| | Technique | | Result |
|---|---|---|---|
| 1st passage | Inject with culture<br>Culture kidney<br>Prepare culture from kidney for 2nd passage | Euthanase at 72h<br>Incubate | >$10^5$ cfu/mL |
| 2nd passage | Inject with culture<br>Culture kidney<br>Prepare culture from kidney for 3rd passage | Euthanase at 72h<br>Incubate | >$10^5$ cfu/mL |
| 3rd passage | Inject with culture<br>Culture kidney<br>Prepare culture from kidney for 4th passage | Euthanase at 72h<br>Incubate | >$10^5$ cfu/mL |
| 4th passage | Inject with culture<br>Culture kidney<br>Collect eye and brain tissue for histology | Euthanase at 7d<br>Incubate | >$10^5$ cfu/mL |

**Table 5.2.5 Summary of the results of tissue sampling for histology**

| Group of fish | Clinical Signs | Brain histology | Eye histology |
|---|---|---|---|
| Kidney tissue transfer | None | Normal | Normal |
| Brain tissue transfer | None | Normal | Normal |
| Culture transfer | Exophthalmia | Normal | Abnormal |

Samples of tissue from the brains showed no lesions. Histological examination of the tissue taken from the fish displaying exophthalmia in either one or both eyes (50% of the remaining fish) showed inflammation characterised by infiltration with macrophages. No bacteria were seen in any of the tissues.

**Discussion**

Although the strain of *Lactococcus garvieae* used for the experiment was not known to be a fish pathogenic strain, since it was isolated from a case of bovine mastitis, it was possible to passage the organism extremely well using the culture technique. For the tissue transfer technique the first passage was performed by injecting the culture and the following passages were performed by injecting homogenised kidney tissue. After the first passage the organism was readily isolated from the kidney tissue, but after the homogenised kidney tissue was injected back into the next groups of fish no growth was apparent on the primary culture plates of the kidney tissue. The organism was able to be reisolated from the enrichment broth. Although, when the experiment was repeated, slightly better results were obtained, there was still a much lower yield than with the culture technique. Additionally, the final passage was extended for 7d to see if any late mortalities arose. Tissue from the brain and eye were sampled for histopathology because, although the organism is found in greater numbers in the kidney, the eye and the brain are the organs most affected by lesions are the eye (ophthalmitis) and brain (meningitis) on gross examination.

Although the bovine isolate of *Lactococcus garvieae* was phenotypically the same as the strain used in experiment one, it was not tested for genotypic similarity by RAPD-PCR as were some other isolates (see Chapter 4.2). The outcome of these two experiments is that both organisms were successfully passaged in rainbow trout using the overnight culture and re-isolation technique. In experiment two, there was no evidence of increased virulence as no mortalities occurred, but the fact that there were some signs of disease in the final extended passage suggests that without the time constraints that existed at the time, there may have been evidence of an increase in virulence. Future work with these organisms may reveal a closer relationship between the two isolates and lends weight to the hypothesis that the fish-associated strains of *Lactococcus garvieae* may, in some instances, have mammalian origins.

## *CHAPTER SIX*: SUMMARY DISCUSSION

This study turned out to be a race against evolving knowledge. Originally the hypothesis was enunciated that streptococcosis of Tasmanian rainbow trout could have been caused by an organism derived from a mammalian source. This hypothesis was prompted by astute observations by a fish farm manager, the preference of the bacterium for high temperatures *in vivo* and *in vitro* and an unsubstantiated report of skin infection in a fish farm employee. At that time the organism was identified by the epithet *Streptococcus* sp. biovar 1, and the bacteria causing streptococcosis in other countries and fish species were poorly characterised.

In the final analysis the work reported in this thesis confirmed that *Streptococcus* sp. biovar 1 consists of two strains of *Lactococcus garvieae* with high pathogenicity for rainbow trout. In contrast, a bovine strain of *Lactococcus garvieae* was found to be almost avirulent to rainbow trout. However, these findings do not necessarily disprove the original hypothesis, as fish-to-fish passage has been shown to considerably enhance the virulence of the piscine isolate of *Lactococcus garvieae* (MP9 {88/3910-15}) and, under appropriate circumstances, similar increases in virulence for fish by the mammalian strains of *Lactococcus garvieae* is feasible. Thus, the final verdict is the Scottish "not proven".

As previously mentioned, another possible route of introduction of *Lactococcus garvieae* could be the feed, as suggested by Bragg & Broere (1986) in relation to outbreaks of an identical disease in South Africa. It has not been unusual to use milk products in trout diets, although there has been no history of such supplements being fed to fish on affected Tasmanian farms (A. Purves pers. comm.). Also, during processing, trout pellets are heated to approximately 100°C (C. Foster pers. comm.), which should be sufficient heat to inactivate *Lactococcus garvieae* which is effectively eliminated by boiling the feed for ten minutes (Bragg & Broere, 1986).

The fact that streptococcosis has not been reported in any other fish species in Tasmania suggests that it is unlikely that the fish-pathogenic strains of *Lactococcus garvieae* originated from a piscine source. However, it is still possible that some other

species could act as an unapparent carrier. Based on local knowledge, the likely carrier species are brown trout, redfin perch, tench or eels. The first mentioned have been demonstrated to be highly resistant to this infection and are not likely to be involved (Munday *et al.* 1993). The disappearance of the disease from previously-affected farms (A. Purves pers. comm.), in the continued presence of redfin perch, tench and eels in the influent waters, does not support involvement of these species. Even so, a survey of these fish for the carrier state would help to further elucidate the epidemiology of *Lactococcus garvieae* infection in Tasmanian rainbow trout.

The fact that *Lactococcus garvieae* only causes disease in fish at water temperatures $\geq 17°C$ and the fact that it is also known to cause disease in cattle suggests that it may have the potential to cause disease in other mammals, including humans. Invasive human infections due to bacterial fish pathogens have been known to occur. Weinstein *et al* (1997) reported that *Streptococcus iniae* was responsible for disease in people who had recently handled fresh fish, mainly tilapia from aquaculture farms. The disease was mainly manifested as cellulitis of the hands although cases of endocarditis have been reported. Human infections have also been reported caused by the fish pathogen *Edwardsiella tarda*, causing symptoms including gastroenteritis, severe wound infections and systemic disease such as septicaemia, meningitis, cholecystitis and osteomyelitis (Janda & Abbott, 1993). Other fish pathogens known to cause human disease include *Vibrio vulnificus* (Amaro & Biosca, 1996; Veenstra *et al*, 1993) and *Aeromonas hydrophila* (Ascencio *et al*, 1991).

Significant advances in knowledge relating to infections of fish caused by Gram-positive cocci were made during the course of this study. For instance, considerable progress was made in developing selective media for *Lactococcus garvieae.* Most of the authors working with strains of *Lactococcus garvieae* had obtained their cultures in a pure form so there is no reference to selective isolation from the environment apart from Bragg *et al.* (1989a) who used selective media that they had developed to try and implicate the leech *Batracobdelloides tricarinata* as the possible vector or reservoir of streptococcosis in rainbow trout in South Africa (Bragg *et al.* 1989b). Kitao *et al.* (1979) used enrichment in a broth containing sodium azide and then subculture onto an *Enterococcus faecalis* medium, which probably also contained

azide, when isolating *Streptococcus* from the seawater near yellowtail culturing pens. However, this streptococcus was found to be quite closely related to what was then known as *Streptococcus faecalis* and their result may not be entirely applicable to *Lactococcus garvieae*.

The selective media reported in this thesis were capable of supporting the growth of *Lactococcus garvieae*, whilst preventing the growth contaminating organisms such as *Aeromonas hydrophila, Pseudomonas aeruginosa,* Methicillin-resistant *Staphylococcus aureus,* yeasts and fungi. This is a significant advance in the microbiological manipulation of this organism and should enable it to be isolated from a variety of environmental sources, even when only present in moderate concentrations. It should be noted, however, that a persistent environmental source seems unlikely as it was not possible to isolate *Lactococcus garvieae* from environmental samples on farms where clinical disease had been reported in rainbow trout in previous years.

The information on phenotypic and genotypic characteristics of the Australian strains of piscine *Lactococcus garvieae* provide pertinent additional data that the organism previously identified as *Streptococcus* sp. biovar 1 (Carson & Munday, 1990 ) and *Enterococcus*-like (Carson *et al.* 1993) is, in fact, two strains of *Lactococcus garvieae* differing in their ability to ferment lactose. Also, the RAPD-PCR technique identified the primer OPB-18 as being particularly suitable for specific identification of *Lactococcus garvieae*, and this may form the basis for a future rapid identification technique not requiring the isolation of the organism in selective media.

It is a perverse fact that clinical streptococcosis of rainbow trout in Tasmania disappeared as a clinical disease at about the time this study commenced. This can probably be attributed to the improved husbandry, especially reduced stocking rates and the complete or partial replacement of susceptible rainbow trout with resistant Atlantic salmon on many farms, including those previously worst affected. However, in view of the explosive nature of the index outbreak, it would be inappropriate if fish farmers became complacent and allowed their management to deteriorate. Even if no carrier fish exist today, the possibility of contamination from a mammalian source

remains. In particular, recent suggestions that yellowtail kingfish, a presumptively highly susceptible species, might be cultured in Australian waters, makes the need for vigilance even more acute.

A number of areas of future research have been identified and these include

1. Development of sensitive PCR techniques for the detection of *Lactococcus garvieae* in fish, feedstuffs and the environment.
2. Surveys of fish in proximity to previously-affected farms, for carrier status for *Lactococcus garvieae*.
3. Surveys of milk products, especially casein, for the presence of *Lactococcus garvieae*.
4. Critical determination of the inactivation temperature of *Lactococcus garvieae*.
5. Attempts to enhance the virulence of mammalian strains of *Lactococcus garvieae* for fish by long-term passage through rainbow trout.

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*APPENDICES*

## APPENDIX A

## MEDIA RECIPES

### A1 Bragg's streptococcal selective agar (BSSA)

| | Grams/litre |
|---|---|
| Agar | 14 |
| Peptone | 10 |
| Lablemco™ | 10 |
| NaCl | 5 |
| Glucose | 10 |
| TTC | 1 |

Mix together all ingredients except triphenyl tetrazolium chloride (TTC) and make up to almost 1 L with distilled water. Sterilise by autoclaving at 121°C for 15min. Prepare solution of TTC and filter sterilise. Mix with other ingredients and make up to 1 L with sterile distilled water and pour plates.

### A2 Bragg's streptococcal selective broth (BSSB)

This recipe has been modified from Bragg's original recipe in that the medium incorporated two of the additives that he experimented with and which displayed greater selectivity in the broth.

| | Grams/litre |
|---|---|
| Nutrient broth | 13 |
| Nalidixic acid | 0.1 |
| Sodium azide | 0.2 |

Mix together above ingredients with 1 L of distilled water and dispense 10 mL aliquots into tubes. Sterilise by autoclaving at 121°C for 15min.

### A3 Modified Streptococcal Selective Agar (MSSA)

Base medium is Columbia Blood Agar Base (Oxoid CM331) prepared by suspending 19.5g in 500 mL of distilled water. Sterilise by autoclaving at 121°C for 15min. Streptococcus Selective Supplement (Oxoid SR126) containing 5mg colistin sulphate and 2.5mg of oxolinic acid and prepared by reconstituting the vial with 2 mL sterile distilled water is added to the sterile 500 mL of Columbia Blood Agar Base. At the

same time add 5 mL of 1% TTC solution to give a final concentration of TTC of 0.01%.

**A4 Modified Streptococcal Selective Broth (MSSB)**

Prepare 6.5g of nutrient broth in 500mL of distilled. Add one vial Streptococcus Selective Supplement (Oxoid SR126) containing 5mg colistin sulphate and 2.5mg of Oxolinic acid and prepared by reconstituting the vial with 2mL sterile distilled water. Dispense 10 mL into tube and sterilise by autoclaving at 121°C for 15min

**A5 Slanetz and Bartley Enterococcus Selective Agar (SBESA)**

Slanetz and Bartley medium (Oxoid CM377) is prepared by suspending 42g in 1L of distilled water. Bring to the boil to dissolve agar completely. Dispense into petri plates.

**A6 Slanetz and Bartley Enterococcus Selective Broth (SBESB)**

| | **Grams/litre** |
|---|---|
| Tryptose | 20 |
| Yeast extract | 5 |
| Glucose | 2 |
| $K_2HPO_4$ | 4 |
| Sodium azide | 0.25 |
| Starch | 10 |

Dissolve ingredients in 1L of distilled water and bring to the boil. Dispense 10mL amounts into sterile tubes.

**A7 Horse blood agar (HBA)**

This is prepared by pouring a 20mL layer of Columbia agar base (Oxoid CM331) into a 90mm petri plate and allowing it to set, followed by a 20mL layer of Columbia agar base containing 7% horse blood.

**A8 Sheep blood agar (SBA)**

This is prepared by pouring a 20mL layer of Columbia agar base (Oxoid CM331) into a 90mm petri plate and allowing it to set, followed by a 20mL layer of Columbia agar base containing 7% sheep blood.

## APPENDIX B

### TESTS AND RECIPES FOR IDENTIFICATION

**B1 Haemolysis**

Incubate on horse blood agar

gamma = no haemolysis

alpha = green zones around colonies

beta = complete clearing of blood around colonies

**B2 Gram stain (Lillie's Modification)**

Crystal Violet

| | |
|---|---|
| Crystal violet | 1 g |
| 95 % alcohol | 20mL |
| 1 % aqueous ammonium oxalate | 80mL |

Leaveovernight. Filter through paper.

Iodine Solution (Jensen's)

| | |
|---|---|
| Iodine crystals | 1g |
| Potassium iodide | 2g |
| Distilled water | 100mL |

Dissolve iodine in strong potassium iodide (2g in 2 mL water) then dilute.

Carbol Fuchsin (Strong)

| | |
|---|---|
| Basic fuchsin (B.D.H.) | 1 g |
| Phenol crystals | 5 g |
| Absolute alcohol | 10mL |
| Distilled water | 100mL |

Steam fuchsin with phenol, add alcohol and water. Filter through paper.

| | |
|---|---|
| 1) Crystal violet 1 % (primary stain) | 30sec |
| 2) Pour off, wash with running tap water | |
| 3) Jensen's iodine (acts as a mordant) | 30sec |

4) Wash in running tap water
5) Decolourise with acetone/alcohol, 70/30, V/V 2sec by holding the slide and pouring the solution over the smear
6) Wash in running water
7) Counterstain with dilute carbol fuchsin 10sec
8) Rinse briefly in running water and blot immediately. Fresh blotting paper must be used between each slide to avoid transfer of organisms from previous slides.
9) Dry high above the Bunsen flame or at room temperature.

**B3 Temperature studies broth**

| | **Amount** |
|---|---|
| Brain heart infusion broth | 25g |
| Glucose | 1g |
| Bromocresol purple 1.6% | 1mL |
| Distilled water | 1L |

1) Dissolve all the ingredients in the water.
2) Dispense 7mL aliquot's into medium sized test tubes
3) Autoclave for 15min at 121$^{o}$C
4) Final pH should be 7.4 ±0.2
5) Check for sterility and quality control

**B4 6.5% Sodium chloride broth**

| | **Amount** |
|---|---|
| Brain heart infusion broth | 25g |
| Sodium chloride | 65g |
| Glucose | 1g |
| Bromocresol purple 1.6% | 1mL |
| Distilled water | 1L |

1) Add the broth and the sodium chloride to the water.
2) Stir to dissolve
3) Dispense 7mL aliquot's into medium sized test tubes
4) Autoclave for 15min at 121$^{o}$C
5) Final pH should be 7.4 ±0.2
6) Check for sterility and quality control

**B5 Bile esculin**

Prepare Oxoid bile esculin (CM888B) by boiling 44.5g/L of media until dissolved, sterilise at 121°C for 15min and pour into petri plates.

**B6 Arginine broth**

| | Grams/litre |
|---|---|
| Tryptone | 5 |
| Yeast extract | 5 |
| Dipotassium hydrogen phosphate | 2 |
| L-arginine monhydrochloride | 3 |
| Dextrose | 0.5 |

Dissolve by heating, adjust pH to 7.0, tube into 5mL amounts and autoclave at 115°C for 10min

**B7 Hippurate hydrolysis**

A 0.4mL aliquot of a 1% solution of sodium hippurate is heavily inoculated with the culture to produce a milky suspension and incubated for 2h at 35°C. Add 0.2mL of ninhydrin reagent and mix well and incubate at 35°C for a further 10min. A deep purple colour indicated a positive test.

**B8 Gelatinase activity**

**Reagents**

| | Grams/Litre |
|---|---|
| Peptone | 4 |
| Yeast extract | 1 |
| Gelatin | 4 |
| Agar | 15 |

Add 1L of distilled water, autoclave and pour in 20mL volumes into petri dishes and allow to set

**Method**

Streak agar with isolated colony of *Streptococcus* spp.

Incubate for 3d at $36^{o}C \pm 1^{o}C$

Flood plates with 15% (w/v) mercuric chloride in 20% (v/v) HCl to enhance cleared

zones around the inoculum (Simbert & Kreig, 1994).

**B9 Acetoin reaction**

| | |
|---|---|
| Tryptone (Oxoid L42) | 20g |
| Yeast extract (Oxoid L 21) | 2g |
| Agar (Oxoid L11) | 15g |
| Distilled water | 900mL |

Dissolve reagents in water. Adjust pH to 7.2. Autoclave at 121°C for 15min. Cool to 50°C. Aseptically add 100mL of 10% 0.2μ filtered glucose and pour 20mL volumes into petri plates (Davis & Hoyling, 1973).

**B10 PYR**

A 0.01% solution of L-pyrrolidonyl-β-naphthylamide is prepared by suspending the amide in distilled water for 24h at 4°C and then aspirating through a 26 gauge needle to emulsify the solution before adding to the broth. An inoculum of 50μL of bacteria equivalent to the McFarland scale 0.5 is added to 50μL of the broth and incubated for 4 hours. Hydrolysis is detected by the addition of 20 μL of 0.5% *p*-dimethylaminocinnameldehyde in 8% v/v concentrated HCl resulting in a change in colour to red.

**B11 Carbohydrate fermentation**

| | |
|---|---|
| Peptone | 10g/L |
| Meat extract | 3g/L |
| NaCl | 5g/L |
| Bromocresol purple | 10mL/L |

Dissolve solids in distilled water, ad the indicator, adjust pH to 7.2. Sterilise at 115°C for 20min. Aseptically add 0.5% of carbohydrate (sterilised by 0.2μ membrane filtration), mix and distribute into sterile tubes.

**B12 Antibiotic sensitivity testing**

**Calibrated Dichotomous Sensitivity Test**

The CDS method is calibrated by dividing organisms into susceptible and resistant categories based on the distribution of their MIC's (hence is dichotomous)

**Media**

Sensitest agar (Oxoid CM409) is prepared with 5% horse blood

**Technique**

1) Use an overnight culture on blood agar
2) Stab 3 - 5 colonies with the sterile tip of a straight wire
3) Emulsify this well in 2.5mL of sterile saline
4) Mix up and down with a sterile Pasteur pipette
5) Flood sensitest agar plate and swirl to ensure complete coverage of the surface
6) Remove excess with pipette
7) Allow plate to air dry for 20-40min
8) Apply antibiotic discs (six discs per plate)
9) Incubate at 35°C overnight in air
10) Measure the zones of inhibition from the back of the plate
11) Measure the annular radius from the edge of the disc to the edge of confluent growth

**Interpretation:**

| | |
|---|---|
| Annular radius | ≥ 6mm = susceptible |
| | < 6mm = resistant |

## APPENDIX C

**Table C1.1 Characteristics differentiating species of *Enterococcus* from Bergey's Manual of Determinative Bacteriology (Holt *et al.* 1994)**

| | *E. avium* | *E. casseliflavus* | *E. cecorum* | *E. dispar* | *E. durans* | *E. faecalis* | *E. faecium* | *E. gallinarum* | *E. hirae* | *E. malodoratus* | *E. mundtii* | *E. pseudoavium* | *E. raffinosus* | *E. saccharolyticus* | *E. seriolicida* | *E. solitarius* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Haemolysis | α | ND | α | ND | α/β | (β) | (α) | α/β | - | ND | - | α | ND | - | α | ND |
| Yellow pigment | - | + | - | - | - | - | - | - | ND | - | + | ND | ND | - | - | - |
| Growth at: | | | | | | | | | | | | | | | | |
| 45°C | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Growth in: | | | | | | | | | | | | | | | | |
| 6.5%NaCl | + | + | - | + | + | + | + | + | + | + | + | - | + | + | + | + |
| Arginine hydrolysis | - | (+) | - | + | + | + | + | + | + | - | + | - | - | - | + | + |
| Hippurate hydrolysis | d | - | - | d | d | (+) | + | + | - | d | - | + | - | - | - | ND |
| VP reaction | - | + | + | ND | ND | ND | ND | ND | + | - | + | + | + | - | + | d |
| Lactose | a | a | a | a | a | a | a | a | a | a | a | a | a | a | - | - |
| Mannitol | a | a | - | - | (-) | a | (a) | a | - | a | a | a | a | a | a | a |
| Raffinose | - | a | a | a | - | - | - | a | d | a | (a) | - | a | a | - | ND |
| Ribose | | | | | | | | | | | | | | | | |
| Sorbitol | a | - | - | - | - | (a) | - | - | - | a | d | a | a | a | a | a |

**Table C1.2 Characteristics differentiating species of *Enterococcus* & *Lactococcus* from API 20S data sheet.**

| | *E. faecalis* 1 | *E. faecalis* 2 | *E. faecalis* 3 | *E. faecium* 1 | *E. faecium* 2 | *E. durans* |
|---|---|---|---|---|---|---|
| Esculin | + | + | + | + | + | + |
| Arginine hydrolysis | + | + | + | + | d | + |
| Hippurate hydrolysis | + | - | + | d | d | d |
| VP/ acetoin reaction | + | + | + | d | d | + |
| PYR | + | + | + | + | + | + |
| Arabinose | - | - | - | a | a | d |
| Lactose | a | a | a | a | a | a |
| Mannitol | a | a | a | a | a | - |
| Raffinose | - | - | - | - | a | - |
| Ribose | a | a | a | a | a | a |
| Sorbitol | a | a | a | - | d | - |
| Starch | a | a | a | d | a | d |
| Trehalose | a | a | a | a | a | a |

| | *E. avium* | *E. gallinarum* | *E. malodoratus* | *L. lactis* | *L. cremoris* | *L. plantarum* |
|---|---|---|---|---|---|---|
| Esculin | + | + | + | + | d | + |
| Arginine hydrolysis | - | + | - | + | - | - |
| Hippurate hydrolysis | d | + | + | d | d | - |
| VP/ acetoin reaction | + | + | + | d | + | + |
| PYR | + | + | + | d | - | - |
| Arabinose | d | a | - | d | - | - |
| Lactose | a | a | a | d | a | - |
| Mannitol | a | a | a | d | d | a |
| Raffinose | d | a | a | - | d | - |
| Ribose | a | a | a | a | d | - |
| Sorbitol | a | - | a | - | - | d |
| Starch | d | a | - | a | d | - |
| Trehalose | a | a | a | a | d | a |

**Table C1.3 Characteristics differentiating species of *Enterococcus* & *Lactococcus* taken from Cowan and Steel's manual for the identification of medical bacteria (Barrow & Feltham, 1993)**

| | *E. faecalis* | *E. faecium* | *E. mundtii* | *E. casseliflavus* | *E. gallinarum* | *E. avium* | *E. durans* | *L. lactis* |
|---|---|---|---|---|---|---|---|---|
| Haemolysis | -/β | α/β | - | α/- | β | α | -/α/β | -/α |
| Yellow pigment | - | - | + | + | - | - | - | - |
| Growth at: 45°C | + | + | + | + | + | + | + | - |
| Growth in: 6.5% NaCl | + | + | + | + | + | + | + | - |
| Growth on 40% Bile Agar | + | + | + | + | + | + | + | + |
| Arginine hydrolysis | + | + | + | + | + | - | + | + |
| Hippurate hydrolysis | d | d | - | - | + | d | d | d |
| Voges-Proskauer | + | + | + | + | + | + | + | + |
| PYR | + | + | + | + | + | + | + | d |
| Arabinose | - | a | a | a | a | d | - | d |
| Lactose | a | a | a | a | a | a | a | d |
| Mannitol | a | a | a | a | a | a | - | d |
| Raffinose | - | d | d | a | a | - | - | - |
| Ribose | a | a | a | a | a | a | a | a |
| Sorbitol | a | d | d | - | - | a | - | - |
| Starch | a | d | - | -/w | a | - | - | a |
| Trehalose | a | a | a | a | a | a | d | d |
| Lancefield antigen | D | D | D | D | D | Q/D | D | N |

**Table C2.1** Characteristics of Tasmanian isolates MP6 to MP38

| | MP6 | MP9 | MP16 | MP17 | MP20 | MP21 | MP28 | MP36 | MP38 |
|---|---|---|---|---|---|---|---|---|---|
| Haemolysis | α | α | α | α | α | α | α | α | α |
| Yellow pigment | - | - | - | - | - | - | - | - | - |
| Growth at: | | | | | | | | | |
| 10°C | + | + | + | + | + | + | + | + | + |
| 45°C | - | - | - | - | - | - | - | - | - |
| Growth in: | | | | | | | | | |
| 6.5% NaCl | + | + | + | + | + | + | + | + | + |
| Bile Esculin | + | + | + | + | + | + | + | + | + |
| Arginine hydrolysis | + | + | + | + | + | + | + | + | + |
| Hippurate hydrolysis | - | - | - | - | - | - | - | - | - |
| Gelatin | - | - | - | - | - | - | - | - | - |
| VP/ acetoin reaction | + | + | + | + | + | + | + | + | + |
| PYR | + | + | + | + | + | + | + | + | + |
| Arabinose | - | - | - | - | - | - | - | - | - |
| Lactose | - | - | a | - | - | - | a | - | a |
| Mannitol | a | a | a | a | a | a | a | a | a |
| Raffinose | - | - | - | - | - | - | - | - | - |
| Ribose | a | a | a | a | a | a | a | a | a |
| Sorbitol | - | - | - | - | - | - | - | - | - |
| Starch | - | - | - | - | - | - | - | - | - |
| Trehalose | a | a | a | a | a | a | a | a | a |
| Agglutination | pos | pos | pos | pos | pos | pos | pos | pos | pos |
| Lancefield group | Not D | Not D | Not D | Not D | Not D | Not D | Not D | Not D | Not D |
| Sensitivity to: | | | | | | | | | |
| Amoxicillin 10 | S | S | S | S | S | S | S | S | S |
| Penicillin 10 | S | S | S | S | S | S | S | S | S |
| Clindamycin 2 | R | R | R | R | R | R | R | R | R |
| Erythromycin 15 | S | S | S | S | S | S | S | S | S |
| Cephalothin 30 | R | R | R | R | R | R | R | R | R |
| Nitrofurantoin 100 | R | R | S | S | R | R | R | R | R |
| Vancomycin 30 | S | S | S | S | S | S | S | S | S |

**Table C2.2** Characterisation results of MP39 to UT9B

| | MP39 | UT2B | UT3B | UT4B | UT5B | UT6B | UT7B | UT8B | UT9B |
|---|---|---|---|---|---|---|---|---|---|
| Haemolysis | α | α | α | α | α | α | α | γ | γ |
| Yellow pigment | - | - | - | + | - | - | - | - | + |
| Growth at: | | | | | | | | | |
| 10°C | + | + | + | + | + | + | + | + | + |
| 45°C | - | - | - | + | + | + | + | + | + |
| Growth in: | | | | | | | | | |
| 6.5% NaCl | + | + | + | + | + | + | + | + | + |
| Bile Esculin | + | + | + | + | + | + | + | + | + |
| Arginine hydrolysis | + | + | + | + | + | + | + | + | + |
| Hippurate hydrolysis | - | - | - | + | + | - | + | + | - |
| Gelatin | - | - | - | - | - | - | - | + | - |
| VP/ acetoin reaction | + | + | + | + | + | + | - | + | + |
| PYR | + | + | + | + | + | + | + | + | + |
| Arabinose | - | - | - | - | - | - | a | - | a |
| Lactose | a | - | - | a | a | a | a | a | a |
| Mannitol | a | a | a | - | - | a | a | a | a |
| Raffinose | - | - | - | - | - | a | a | - | a |
| Ribose | a | a | a | a | a | a | a | a | a |
| Sorbitol | - | - | - | - | - | - | - | a | - |
| Starch | - | - | - | a | a | a | a | a | - |
| Trehalose | a | a | a | a | a | a | a | a | a |
| Agglutination | pos | pos | pos | neg | neg | neg | neg | neg | neg |
| Lancefield group | Not D | Not D | Not D | D | D | D | D | D | D |
| Sensitivity to: | | | | | | | | | |
| Amoxicillin 10 | S | S | S | S | S | S | S | S | S |
| Penicillin 10 | S | S | S | S | S | S | S | S | S |
| Clindamycin 2 | R | R | R | R | R | R | R | R | R |
| Erythromycin 15 | S | S | S | S | S | R | S | S | R |
| Cephalothin 30 | R | R | R | R | R | R | R | R | R |
| Nitrofurantoin 100 | R | R | R | R | R | R | R | S | R |
| Vancomycin 30 | S | S | S | S | S | S | S | S | S |

**Table C2.3** Characterisation results on UT1C to UT9C

| | UT1C | UT2C | UT3C | UT4C | UT5C | UT6C | UT7C | UT8C | UT9C |
|---|---|---|---|---|---|---|---|---|---|
| Haemolysis | α | α | γ | α | α | γ | α | β | γ |
| Yellow pigment | - | - | + | - | - | - | + | - | - |
| Growth at: | | | | | | | | | |
| 10°C | + | + | + | + | + | + | + | + | + |
| 45°C | - | + | + | + | + | + | + | + | - |
| Growth in: | | | | | | | | | |
| 6.5% NaCl | + | + | + | + | + | + | + | + | + |
| Bile Esculin | + | + | + | + | + | + | + | + | + |
| Arginine hydrolysis | - | + | + | + | + | + | + | + | + |
| Hippurate hydrolysis | - | + | - | + | + | + | - | + | - |
| Gelatin | - | - | - | - | - | + | - | - | - |
| VP/ acetoin reaction | + | + | + | + | + | + | + | + | + |
| PYR | - | + | + | - | - | + | + | + | - |
| Arabinose | - | a | a | - | - | - | a | - | a |
| Lactose | a | a | a | a | a | a | a | a | a |
| Mannitol | a | a | a | a | a | a | a | a | a |
| Raffinose | a | - | - | - | - | - | - | - | - |
| Ribose | a | a | a | a | a | a | a | a | a |
| Sorbitol | a | - | a | a | a | a | a | a | - |
| Starch | - | a | - | - | - | a | - | a | a |
| Trehalose | a | a | a | a | a | a | a | a | a |
| Agglutination | w | neg | neg | neg | neg | neg | neg | neg | neg |
| Lancefield group | Not D | D | D | D | D | D | D | D | Not D |
| Sensitivity to: | | | | | | | | | |
| Amoxicillin 10 | R | S | R | S | S | S | S | S | S |
| Penicillin 10 | R | R | R | S | S | S | S | S | S |
| Clindamycin 2 | S | R | S | R | R | R | R | R | S |
| Erythromycin 15 | S | R | R | R | R | R | S | S | S |
| Cephalothin 30 | R | R | R | R | R | R | R | R | S |
| Nitrofurantoin 100 | R | R | R | S | S | S | S | S | S |
| Vancomycin 30 | S | S | S | S | S | S | S | S | S |

**Table C2.4** Characterisation results for samples UT1D to UT9D

| | UT1D | UT2D | UT3D | UT4D | UT5D | UT6D | UT7D | UT8D | UT9D |
|---|---|---|---|---|---|---|---|---|---|
| Haemolysis | γ | β | β | α | γ | γ | γ | γ | α |
| Yellow pigment | - | - | - | - | - | - | - | - | - |
| Growth at: | | | | | | | | | |
| 10°C | + | + | + | + | + | + | + | + | + |
| 45°C | - | - | + | + | + | - | - | + | - |
| Growth in: | | | | | | | | | |
| 6.5% NaCl | - | - | - | - | + | + | - | + | + |
| Bile Esculin | + | + | + | + | + | - | + | + | - |
| Arginine hydrolysis | + | - | - | - | + | | + | + | + |
| Hippurate hydrolysis | + | - | - | - | + | w | - | + | + |
| Gelatin | - | - | - | - | - | - | - | + | - |
| VP/ acetoin reaction | + | + | + | + | + | + | + | + | - |
| PYR | + | - | - | + | + | - | - | + | + |
| Arabinose | - | - | - | a | a | a | a | - | - |
| Lactose | - | - | - | a | a | a | a | a | - |
| Mannitol | a | a | a | a | a | a | a | a | a |
| Raffinose | - | - | - | a | - | - | - | - | - |
| Ribose | a | a | a | a | a | a | a | a | a |
| Sorbitol | - | a | a | - | - | - | - | a | - |
| Starch | a | a | a | a | a | a | a | a | - |
| Trehalose | a | a | a | a | - | a | a | a | a |
| Agglutination | neg | pos | neg | neg | neg | w | neg | neg | neg |
| Lancefield group | Not D | Not D | D | D | D | Not D | Not D | D | D |
| Sensitivity to: | | | | | | | | | |
| Amoxicillin 10 | S | S | S | S | S | S | S | S | S |
| Penicillin 10 | S | S | S | S | R | S | S | S | S |
| Clindamycin 2 | R | R | R | R | R | S | S | R | R |
| Erythromycin 15 | S | S | R | R | S | S | S | R | S |
| Cephalothin 30 | R | R | R | R | R | S | S | R | S |
| Nitrofurantoin 100 | R | S | R | S | R | S | R | S | S |
| Vancomycin 30 | S | S | R | R | S | S | S | S | S |

**Table C2.5** Characterisation results of samples UT1E to UT9E

| | UT1E | UT2E | UT3E | UT4E | UT5E | UT6E | UT7E | UT8E | UT9E |
|---|---|---|---|---|---|---|---|---|---|
| Haemolysis | α | α | α | γ | α | γ | γ | α | γ |
| Yellow pigment | - | + | - | - | + | - | - | - | - |
| Growth at: | | | | | | | | | |
| 10°C | + | + | + | + | + | + | + | + | + |
| 45°C | + | + | + | - | + | + | - | - | - |
| Growth in: | | | | | | | | | |
| 6.5% NaCl | + | + | + | - | + | + | - | - | + |
| Bile Esculin | + | + | + | + | + | + | + | + | + |
| Arginine hydrolysis | + | + | + | + | + | + | + | - | + |
| Hippurate hydrolysis | - | - | - | + | - | + | - | - | - |
| Gelatin | - | - | - | - | - | + | - | - | - |
| VP/ acetoin reaction | + | + | + | + | + | + | + | - | + |
| PYR | + | + | + | - | + | + | - | - | - |
| Arabinose | a | a | a | - | a | - | a | - | a |
| Lactose | a | a | a | a | a | - | a | - | a |
| Mannitol | a | a | a | a | a | a | a | - | a |
| Raffinose | - | - | a | - | a | - | - | - | - |
| Ribose | a | a | a | a | a | a | a | a | a |
| Sorbitol | a | a | - | - | - | a | - | - | - |
| Starch | a | - | a | a | a | a | a | - | - |
| Trehalose | a | a | a | a | a | a | a | a | a |
| Agglutination | neg | neg | neg | pos | neg | neg | pos | w | neg |
| Lancefield group | D | D | D | Not D | D | D | Not D | Not D | Not D |
| Sensitivity to: | | | | | | | | | |
| Amoxicillin 10 | S | S | S | S | S | S | S | S | S |
| Penicillin 10 | S | S | S | S | S | S | S | S | S |
| Clindamycin 2 | R | R | R | S | R | R | S | R | S |
| Erythromycin 15 | S | S | R | S | R | R | S | S | S |
| Cephalothin 30 | R | R | R | S | R | R | S | S | S |
| Nitrofurantoin 100 | S | S | S | S | S | S | R | R | S |
| Vancomycin 30 | S | S | S | S | S | S | S | S | S |

**Table C2.6** Characterisation results of samples UT1F to UT9F

| | UT1F | UT2F | UT3F | UT4F | UT5F | UT6F | UT7F | UT8F | UT9F |
|---|---|---|---|---|---|---|---|---|---|
| Haemolysis | α | γ | γ | γ | γ | γ | γ | α | α |
| Yellow pigment | - | - | - | - | - | - | - | - | - |
| Growth at: | | | | | | | | | |
| 10°C | + | + | + | + | + | + | + | + | + |
| 45°C | + | + | + | + | + | - | - | - | - |
| Growth in: | | | | | | | | | |
| 6.5% NaCl | + | + | + | + | + | + | + | - | + |
| Bile Esculin | + | + | + | + | + | + | + | + | + |
| Arginine hydrolysis | + | + | + | + | + | + | + | - | - |
| Hippurate hydrolysis | + | - | - | + | w | + | + | - | - |
| Gelatin | - | - | - | + | - | - | - | - | - |
| VP/ acetoin reaction | + | + | + | + | + | + | + | + | + |
| PYR | + | - | - | + | - | + | + | - | - |
| Arabinose | a | a | a | - | a | - | - | - | - |
| Lactose | a | a | a | a | a | a | a | a | a |
| Mannitol | a | a | a | a | a | a | a | a | a |
| Raffinose | a | - | - | - | a | - | - | a | a |
| Ribose | a | a | a | a | a | a | a | a | a |
| Sorbitol | a | - | - | a | - | - | - | a | a |
| Starch | a | a | a | - | a | a | a | - | - |
| Trehalose | a | a | a | a | a | a | a | a | a |
| Agglutination | neg | neg | neg | neg | neg | neg | neg | neg | neg |
| Lancefield group | D | D | D | D | D | D | Not D | Not D | Not D |
| Sensitivity to: | | | | | | | | | |
| Amoxicillin 10 | R | S | S | S | S | R | R | R | R |
| Penicillin 10 | R | S | S | S | S | R | R | R | R |
| Clindamycin 2 | R | R | R | R | S | R | R | R | R |
| Erythromycin 15 | R | S | S | R | S | S | S | S | S |
| Cephalothin 30 | R | R | R | R | S | R | R | R | R |
| Nitrofurantoin 100 | R | S | S | S | R | S | S | S | R |
| Vancomycin 30 | S | S | S | S | S | S | S | S | S |

**Table C2.7** Characterisation results of samples UT1G to UT9G

| | UT1G | UT2G | UT3G | UT4G | UT5G | UT6G | UT7G | UT8G | UT9G |
|---|---|---|---|---|---|---|---|---|---|
| Haemolysis | γ | α | α | α | α | α | α | γ | γ |
| Yellow pigment | - | - | - | - | - | - | - | - | - |
| Growth at: | | | | | | | | | |
| 10°C | + | + | + | + | + | -/w | + | + | + |
| 45°C | + | - | + | + | + | + | + | + | + |
| Growth in: | | | | | | | | | |
| 6.5% NaCl | + | + | + | + | + | + | + | + | + |
| Bile Esculin | + | + | + | + | + | + | + | + | + |
| Arginine hydrolysis | + | + | + | + | + | + | + | + | + |
| Hippurate hydrolysis | + | - | + | - | w | - | + | + | + |
| Gelatin | - | - | - | - | - | - | - | + | - |
| VP/ acetoin reaction | + | + | + | + | + | + | + | + | + |
| PYR | + | + | + | + | + | + | + | + | + |
| Arabinose | a | a | a | - | - | a | - | - | - |
| Lactose | a | a | a | a | a | a | a | a | a |
| Mannitol | a | a | a | a | a | a | - | a | a |
| Raffinose | a | a | a | - | - | a | a | - | - |
| Ribose | a | a | a | a | a | a | a | a | a |
| Sorbitol | a | a | a | a | - | a | - | a | - |
| Starch | a | a | a | a | a | a | a | a | a |
| Trehalose | a | a | a | a | a | a | a | a | a |
| Agglutination | neg | pos | neg | neg | neg | neg | neg | neg | neg |
| Lancefield group | D | Not D | D | D | D | D | D | D | D |
| Sensitivity to: | | | | | | | | | |
| Amoxicillin 10 | R | S | S | S | S | S | S | S | S |
| Penicillin 10 | R | S | S | S | S | S | S | S | S |
| Clindamycin 2 | R | R | S | R | R | R | R | R | S |
| Erythromycin 15 | R | S | S | S | S | R | S | R | S |
| Cephalothin 30 | R | R | R | R | R | R | R | R | R |
| Nitrofurantoin 100 | R | S | R | R | R | S | S | S | S |
| Vancomycin 30 | S | S | S | S | S | S | S | S | S |

**Table C2.8** Characterisation results on samples UT1H to UT9H

| | UT1H | UT2H | UT3H | UT4H | UT5H | UT6H | UT7H | UT8H | UT9H |
|---|---|---|---|---|---|---|---|---|---|
| Haemolysis | γ | γ | γ | γ | β | γ | γ | α | α |
| Yellow pigment | - | - | - | - | - | - | - | - | - |
| Growth at: | | | | | | | | | |
| 10°C | + | + | + | + | + | + | + | + | |
| 45°C | + | + | + | + | + | + | + | - | + |
| Growth in: | | | | | | | | | |
| 6.5% NaCl | + | + | + | + | + | + | + | - | - |
| Bile Esculin | + | + | + | + | + | + | + | + | - |
| Arginine hydrolysis | + | + | + | + | + | + | + | - | |
| Hippurate hydrolysis | - | - | - | + | + | - | + | - | - |
| Gelatin | - | - | - | - | - | - | - | - | - |
| VP/ acetoin reaction | + | + | + | + | + | + | + | - | - |
| PYR | + | + | + | + | + | + | + | - | + |
| Arabinose | a | a | a | - | - | a | - | - | - |
| Lactose | a | a | a | a | a | a | a | - | a |
| Mannitol | a | a | a | a | - | a | - | a | a |
| Raffinose | - | - | a | - | - | - | - | - | - |
| Ribose | a | a | a | a | a | a | a | a | a |
| Sorbitol | a | a | - | a | - | a | - | a | - |
| Starch | a | a | a | a | a | a | a | a | - |
| Trehalose | a | a | a | a | a | a | - | a | a |
| Agglutination | neg | neg | neg | neg | neg | neg | neg | neg | w |
| Lancefield group | D | D | D | D | D | D | D | D | Not D |
| Sensitivity to: | | | | | | | | | |
| Amoxicillin 10 | S | S | S | S | S | S | S | S | S |
| Penicillin 10 | S | S | S | S | S | S | S | S | S |
| Clindamycin 2 | R | R | R | R | R | R | S | R | R |
| Erythromycin 15 | S | S | S | S | S | S | S | R | S |
| Cephalothin 30 | R | S | R | R | R | R | R | R | S |
| Nitrofurantoin 100 | S | S | S | S | S | S | S | S | S |
| Vancomycin 30 | S | S | S | S | S | S | S | S | S |

**Table C2.9** Characterisation results of samples UT1I, UT2I, MPLG1 & MPLG2

| | UT1I | UT2I | MPLG1 | MPLG2 |
|---|---|---|---|---|
| Haemolysis | α | α | α | α |
| Yellow pigment | - | - | - | - |
| Growth at: | | | | |
| 10°C | - | + | + | + |
| 45°C | - | - | - | - |
| Growth in: | | | | |
| 6.5% NaCl | - | + | + | + |
| Bile Esculin | - | + | + | - |
| Arginine hydrolysis | + | + | + | + |
| Hippurate hydrolysis | w? | - | - | - |
| Gelatin | - | - | - | - |
| VP/ acetoin reaction | - | + | + | + |
| PYR | + | + | + | + |
| Arabinose | - | - | - | - |
| Lactose | a | a | - | - |
| Mannitol | a | a | a | a |
| Raffinose | - | - | - | - |
| Ribose | a | a | a | a |
| Sorbitol | - | - | - | - |
| Starch | - | - | - | - |
| Trehalose | a | a | a | a |
| Agglutination | neg | pos | pos | w |
| Lancefield group | Not D | Not D | Not D | Not D |
| Sensitivity to: | | | | |
| Amoxicillin 10 | S | S | S | S |
| Penicillin 10 | S | S | S | S |
| Clindamycin 2 | R | R | R | R |
| Erythromycin 15 | S | S | S | S |
| Cephalothin 30 | S | R | R | R |
| Nitrofurantoin 100 | S | R | R | R |
| Vancomycin 30 | S | S | S | S |

## APPENDIX D

**Standard spread plate method for counting bacteria**

1) Prepare the injectant in PBS
2) Make serial x10 dilutions, usually $10^{-3}$ to $10^{-6}$
3) Pipette 0.1mL aliquots into the surface of HBA which have been appropriately labelled
4) Spread the inoculum over the surface of the agar using a sterile glass spreader. The glass spreader is sterilised by dipping into and then flaming.
5) Invert plates and incubate aerobically overnight at 35 °C.
6) Count the number of colonies on the plates
7) Calculate the number of viable bacteria by multiplying colony count of the plate by the dilution factor and multiply this by 10.

Colony count x dilution x 10 = number of colony forming units (cfu)/mL